Volume 22 May 1958 No. 3

BULLETIN OF THE AGRICULTURAL CHEMICAL SOCIETY OF JAPAN

ANALYTICAL CHEMISTRY ORGANIC CHEMISTRY BIOLOGICAL CHEMISTRY
FOOD & NUTRITION MICROBIOLOGY & FERMENTATION INDUSTRY
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CONTENTS

Organic Chemistry

K. YAGISHITA: The Chemistry of Ilexol. III. Isomerization of Ilexol and its Derivatives. 131

YS. CHEN: Studies on Metabolic Products of *Hypochnus sasakii* Shirai. Isolation of *p*-Hydroxyphenyl Acetic Acid and its Physiological Activity. .. 136

T. SUGITA, Y. INOUE and M. OHNO: Geometrical Isomers of α-Methylmuconic Acid.......... 162

Y. KATSUDA, T. CHIKAMOTO and Y. INOUE: Relationship between Stereoisomerism and Biological Activity of Pyrethroids. Part I. Synthesis of Homochrysanthemic Acid and Related Compounds. 185

Biological Chemistry

I. YAMASAKI, S. UEDA and C. TODA: On the Susceptibility of the Crystalline and Retrograded Forms of Amylose of Different Molecular Weights toward Black-koji Amylase System.......... 157

D. TSURU, T. YAMAMOTO and J. FUKUMOTO: Amylase Formation by Lysozyme Lysate of *Bac. subtilis*.......... 168

M. NAGASAWA: Shikimic Acid in the Tobacco Leaf. 174

Y. SATOMURA, S. OI and A. SAWADA: Intracellular Lipase Formation by Washed Mycelium. (Biochemical Studies on *Sclerotiana libertiana*, Part 13) .. 194

Food and Nutrition

Y. OBATA and H. HORITSU: Studies on the Sunlight Flavour of Beer. Part V. The Genetical Conditions of Occurrence of the Sunlight Flavour of Beer.......... 153

Microbiology and Industry Fermentation

H. KATAGIRI and T. TOCHIKURA: Microbiological Studies of *Coli-aerogenes* Bacteria. Part V. A Cyclic Mechanism of Aerobic Carbon-Metabolism. .. 143

S. KINOSHITA, K. NAKAYAMA and S. AKITA: Taxonomical Study of Glutamic Acid Accumulating Bacteria, *Micrococcus glutamics* nov. sp. 176

(*continued on third page of cover*)

Published by the Agricultural Chemical Society of Japan
c/o Faculty of Agriculture, University of Tokyo

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Manuscripts submitted for publication and all inquiries regarding them should be sent to the Editor of the Bulletin, The Agricultural Chemical Society of Japan, c/o Departments of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo, Bunkyo-ku, Tokyo, Japan.

Annual subscription price is $6.00 for all oversea subscribers including postage fee. Please address orders to the agencies.

The Agricultural Chemical Society of Japan also published the Journal of the Agricultural Chemical Society of Japan (Nippon Nogei Kagakukaishi), printed in Japanese.

AGENCIES

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P. O. Box 605, Tokyo Central
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[Bull. Agr. Chem. Soc. Japan, Vol. 22, No. 3, p. 131~136, 1958]

The Chemistry of Ilexol. III.*

Isomerization of Ilexol and its Derivatives

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Received December 20, 1957

It has now been shown that: (a) treatment with either perchloric acid or sulfuric acid of ilexol acetate in a mixture of benzene and ethanol, and (b) treatment with platinum-catalyzed hydrogen of ilexol and ilexone, affords an isomeric alcohol designated as isoilexol, which has $C_{30}H_{50}O$, m.p. 177~178°, $[\alpha]_D^{20}+41.66°$ (acetate, $C_{32}H_{52}O_2$, m.p. 226~227°, $[\alpha]_D^{27}$ $+48.92°$).

Furthermore, it has been shown that: (a) treatment with hydrochloric acid and glacial acetic acid of ilexol acetate affords α-amyrin acetate, and, in addition, an acetate (Acetate A), $C_{32}H_{52}O_2$, m.p. 212~213°, $[\alpha]_D^{19}-22.70°$, which is hydrolyzed to an alcohol (Alcohol A), $C_{30}H_{50}O$, m.p. 202~203°, $[\alpha]_D^{19}-35.88°$; (b) treatment with sulfuric acid and glacial acetic acid of ilexol acetate and (c) treatment with hydrochloric acid and glacial acetic acid of isoilexol acetate, affords only Acetate A respectively.

The infrared and ultraviolet spectra of these isomerized compounds have been measured, and the results of which are discussed.

In the preceding paper[1] of this series, it has been shown that ilexol is a triterpenoid alcohol (formation of ilexone and ilexene), the secondary alcoholic hydroxyl group of which might be attached to the C-3 atom in its molecule.

The present paper deals with the results of experiments, which were carried out on isomerization of ilexol and its derivatives using various isomerizing agents.

It has now been shown that treatment with either perchloric acid or sulfuric acid of ilexol acetate suspended in a mixture of ethanol and benzene, affords an isomeric alcohol designated as isoilexol, which has $C_{30}H_{50}O$, m.p. 177~178°, $[\alpha]_D^{20}+41.66°$ (c, 0.512), and gives an acetate, $C_{32}H_{52}O_2$, m.p. 226~227°, $[\alpha]_D^{27}+48.92°$ (c, 0.511).

Hereby, it is noteworthy that treatment with platinum-catalyzed hydrogen[2] of ilexol and ilexone suspended in a mixture of glacial acetic acid and cyclohexane, gives rise to one and the same isomeric alcohol identical with isoilexol.

Furthermore, it has now been shown that treatment with hydrochloric acid and glacial acetic acid[3] of ilexol acetate affords: (a) an acetate (Acetate A), $C_{32}H_{52}O_2$, m.p. 212~213°, $[\alpha]_D^{19}-22.70°$ (c, 0.488), which is hydrolyzed to an alcohol (Alcohol A), $C_{30}H_{50}O$, m.p. 202 ~203°, $[\alpha]_D^{20}-35.88°$ (c, 0.385), and (b) an acetate, $C_{32}H_{52}O_2$, m.p. 222~224°, $[\alpha]_D^{20}+76.35°$ (c, 0.572), which is hydrolyzed to α-amyrin,

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* Part II: This Bulletin, **22**, 123 (1958). Presented at the General Meeting of the West Japan Branch of the Agricultural Chemical Society of Japan held Nov. 16, 1957 at Kyushu University, Fukuoka, Japan.

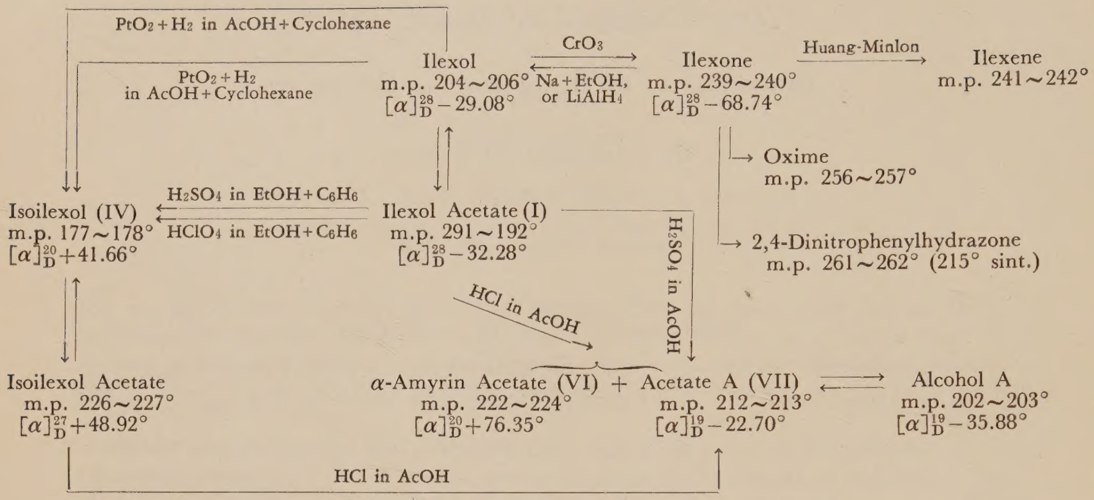
** Ooemachi, Kumamoto City, Japan.

[1] K. Yagishita and S. Iseda, This Bulletin, **21**, 274 (1957)

[2] cf. H. Wieland and W. Benend, *Ann.*, **554**, 1 123 (1943); H. E. Stavely and G. H. Bollenback, *J. Am. Chem. Soc.*, **65**, 1600 (1943); D. E. R. Barton and J. D. Cox, *J. Chem. Soc.*, **1948**, 1354.

[3] cf. J. M. Beaton, F. S. Spring, R. Stevenson and J. L. Stewart, *J. Chem. Soc.*, **1955**, 2131.

CHART I



indicative of the fact that ilexol might well be a triterpenoid alcohol of the α-amyrin group.

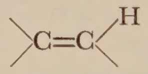
Differing, however, from the foregoing result, it has now been shown that treatment with sulfuric acid and glacial acetic acid of ilexol acetate affords only one acetate identical with the foregoing Acetate A.

As expected, it has also been shown that treatment with hydrochloric acid and glacial acetic acid of isoilexol acetate, affords only one acetate identical with the foregoing Acetate A, indicative of the fact that isoilexol is an intermediate compound between ilexol and Alcohol A.

TABLE I

Compound	m. p.	Optical Rotation
Ursa-13(18)-en-3β-ol[4]	201~202°	[α]$_D$ −36°, −37°
Ursa-13(18)-en-3β-ol[5]	204~205°	[α]$_D$ −35°, −36°
"Alcohol A"	202~203°	[α]$^{19}_{D}$ −35.88°
Ursa-13(18)-en-3β-yl Acetate[4]	213~215°	[α]$_D$ −22°
Ursa-13(18)-en-3β-yl Acetate[5]	214~216°	[α]$_D$ −22°, −23°
"Acetate A"	212~213°	[α]$^{19}_{D}$ −22.70°

As shown in the Table I, the melting points and values of rotation of Alcohol A and Acetate A closely resemble those[4],[5] of ursa-13(18)-en-3β-ol and ursa-13(18)-en-3β-yl acetate respectively, suggestive of their close structural interrelationship.

Although each of the infrared spectra of ilexol, ilexol acetate, ilexone and ilexene[1] reveals a sharp absorption band at 819, 820, 817 and 817 cm^{-1} characteristic of the grouping  $>C{=}C<^{H}$ each, it has now been shown that the infrared spectra of both isoilexol and its acetate do not reveal any appreciable absorption band in this region respectively, indicative of the disappearance of the above grouping in each of their molecules.

Hereby, it is also to be noted that ilexol and each of its derivatives obtained so far, exhibit a positive color reaction with tetranitromethane each, indicative of the presence of at least one double linkage in each of their molecules.

---

[4] J. M. Beaton, F. S. Spring, R. Stevenson and W. S. Strachan, *J. Chem. Soc.*, **1955**, 2610.

[5] J. D. Easton, W. Manson and F. S. Spring, *ibid.*, **1953**, 943.

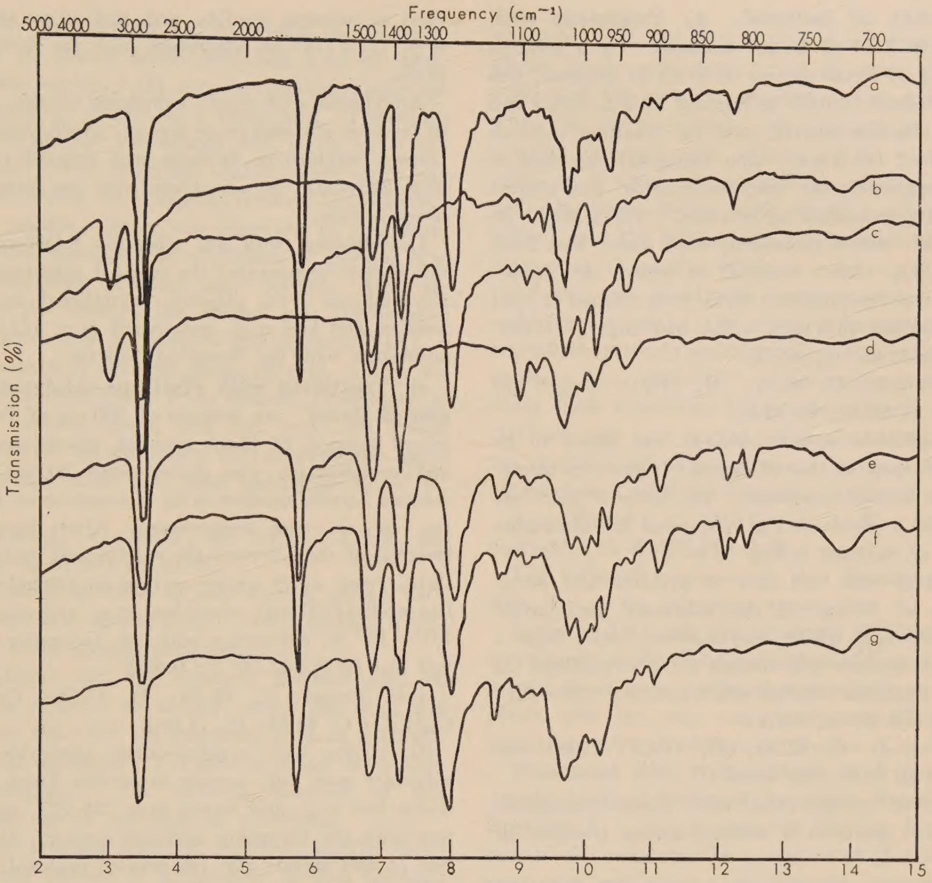


FIG. 1.

a) Ilexol Acetate; b) Ilexol; c) Isoilexol Acetate; d) Isoilexol; e) α-Amyrin Acetate; f) α-Amyrin Acetate (from Ilexol Acetate); g) "Acetate A".

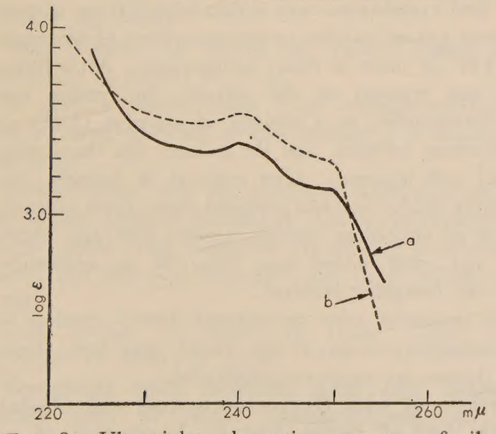


FIG. 2. Ultraviolet absorption spectra of ilexol (a: ——), and isoilexol (b: ------).

EXPERIMENTAL*

Color Test with Tetranitromethane of Ilexol, Ilexol Acetate, Ilexone and Ilexene. The color tests were carried out in the usual manner, and the following results were obtained.

Ilexol: yellow; ilexol acetate: yellow; ilexone: faintly orange; ilexene: orange.

---

* Unless otherwise stated, a) all melting points were corrected; b) rotations were measured in $CHCl_3$ solution, using 1-dm tubes; c) infrared spectra were measured in a Nujol mull with a Perkin-Elmer Model 21 double-bea minstrument equipped with rock-salt prisms; d) ultraviolet spectra were measured in cyclohexane solution with a Beckman DU ultraviolet spectrophotometer; e) adsorption chromatography was carried out using the column of alumina (200 mesh in size) purchased from Wako Pure Chemicals Co., and petroleum ether (b.p. 60~80°).

Formation of Isoilexol. a) Treatment with Perchloric Acid of Ilexol Acetate. To a solution of 400 mg of ilexol acetate in 20 ml of benzene, was added a mixed solution of 8 ml of $HClO_4$ (60%) and 70 ml of absolute ethanol, and the resulting mixture was refluxed for 6 hrs. After being left overnight at room temperature, the reaction mixture was diluted with water, and taken up in ether. The ethereal solution was washed thoroughly with water, and dried with Na_2SO_4. After removal of ether, the residue (380 mg) was recrystallized thrice from ethanol to yield colorless needles (350 mg), which had m.p. 177~178° and $[\alpha]_D^{20}+41.66°$ (c, 0.512).

Anal. Found: C, 84.35; H, 11.91. Calcd. for $C_{30}H_{50}O$: C, 84.44; H, 11.81.

Its solubility in organic solvents was found to be almost the same as that of ilexol. It gave an orange color with tetranitromethane. The infrared spectrum of isoilexol is shown in Fig. 1, d., and the ultraviolet spectrum of isoilexol in Fig. 2, b.

Acetylation with 1 ml each of pyridine and acetic anhydride of 200 mg of this substance gave crude acetate (190 mg), which, recrystallized thrice from a mixture of benzene and ethanol (1:1), crystallized as colorless, prismatic needles, which had m.p. 226~227° and $[\alpha]_D^{27}+48.92°$ (c, 0.511).

Anal. Found: C, 82.20; H, 11.42. Calcd. for $C_{32}H_{52}O_2$: C, 81.99; H, 11.18.

It gave an orange color with tetranitromethane. The infrared spectrum of isoilexol acetate is shown in Fig. 1,c.

Deacetylation with 3% ethanolic KOH-solution of 80 mg of this acetate regenerated the original substance, which had m.p. and mixed m.p. 177~178° on admixture with isoilexol obtained as above.

b) Treatment with Sulfuric Acid of Ilexol Acetate. To a suspension of 200 mg of ilexol acetate in 10 ml of benzene, was added a mixture of 1 ml of H_2SO_4 (d, 1.84) and 22 ml of absolute ethanol, and the resulting mixture was refluxed for 5 hrs. on a water-bath.

After dilution with cold water, the reaction mixture was taken up in ether, and the ethereal solution was thoroughly washed with water, and dried. After removal of ether, the residue was dissolved in 30 ml of a mixture of petroleum ether and benzene (5:2), and the resulting solution was chromatographed on a column of alumina (2×6 cm). Elution with a mixture of chloroform-methanol (1:1) off the column gave a fraction (160 mg), which, recrystallized several times from a mixture of chloroform and methanol (1:1), crystallized as colorless needles, and had m.p. and mixed m.p. 177~178° on admixture with the foregoing isoilexol.

Acetylation with acetic anhydride of this substance in benzene afforded crude acetate, which, recrystallized from a mixture of benzene and ethanol (1:1), had m.p. 226~227° on admixture with the foregoing isoilexol acetate.

Deacetylation with 3% ethanolic KOH-solution of this acetate regenerated the original substance, which, recrystallized from ethanol, crystallized as colorless needles, and had m.p. and mixed m.p. 177~178° on admixture with the foregoing isoilexol.

c) Treatment with Platinum-catalyzed Hydrogen of Ilexol. An amount of 200 mg of ilexol in a mixed solution of 15 ml each of glacial acetic acid and cyclohexane, was shaken with 200 mg of pre-reduced Adams' catalyst in an atmosphere of hydrogen for 1 hr. at room temperature. After filtration and removal of the solvents, the residue was recrystallized thrice from ethyl acetate, giving an alcohol as colorless needles (170 mg), which had m.p. and mixed m.p. 177~178° on admixture with the foregoing isoilexol, and had $[\alpha]_D^{20}+42.15°$ (c, 0.422).

Anal. Found: C, 84.23; H, 11.75. Calcd. for $C_{30}H_{50}O$: C, 84.44; H, 11.81.

Acetylation with pyridine-acetic anhydride of this substance gave pure acetate as needles from ethanol, which had m.p. and mixed m.p. 225–227° on admixture with the foregoing isoilexol acetate. Deacetylation of this acetate also regenerated isoilexol.

d) Treatment with Platinum-catalyzed Hydrogen of Ilexone. An amount of 200 mg of ilexone in a mixed solution of 15 ml each of glacial acetic acid and cyclohexane was shaken with 200 mg of pre-reduced Adams' catalyst in an atmosphere of hydrogen for 1 hr. or more at room temperature. After filtration and removal of the solvent, the residue was chromatographed on a column of alumina (2×6 cm) in benzene solution, and the column was thoroughly eluted with benzene. After removal of benzene, the resulting residue was recrystallized thrice from ethanol, giving an alcohol as colorless needles (160 mg), which had m.p. and mixed m.p. 177~178° on admixture with the foregoing isoilexol.

On treatment with pre-reduced Adams' catalyst in an atmosphere of air, it was found that both ilexol and ilexone are recovered unaffected.

Treatment with Hydrochloric Acid and Glacial Acetic Acid of Ilexol Acetate—Formation of α-Amyrin Acetate and Acetate A. An amount of

200 mg of ilexol acetate was suspended in a mixture of 38 ml of glacial acetic acid and 2 ml of conc. hydrochloric acid (d, 1.19), and the resulting suspension was refluxed for 17 hrs. on a water-bath. The resulting reaction mixture was diluted with water and taken up in ether. After thorough washing with water and subsequent drying with Na_2SO_4, removal of the solvent gave the residue, which was dissolved in petroleum ether, chromatographed on a column of alumina (2×6 cm), and eluted with the same solvent. After removal of the solvent, the residue was fractionated using a mixture of chloroform and methanol (1:1).

There separated colorless plates (90 mg), which, recrystallized several times from ethanol, crystallized as colorless, lustrous plates, and had m.p. and mixed m.p. 222~224° on admixture with an authentic specimen of α-amyrin acetate[6], and had $[\alpha]_D^{20}+76.35°$ (c, 0.752).

Anal. Found: C, 82.12; H, 11.38. Calcd. for $C_{32}H_{52}O_2$: C, 81.99; H, 11.18.

The infrared spectrum of this acetate agreed with that of α-amyrin acetate (Fig. 1, e~f). It gave a yellow color with tetranitromethane.

Deacetylation with 3% ethanolic KOH-solution of this acetate afforded an alcohol, which had m.p. and mixed m.p. 186~187° on admixture with an authentic specimen of α-amyrin[6]. It gave a yellow color with tetranitromethane. The infrared spectrum of this alcohol also agreed with that of α-amyrin.

From the fraction freed of α-amyrin acetate, there was obtained Acetate A as colorless needles (70 mg), which, recrystallized several times from a mixture of chloroform and methanol (1:1), crystallized as colorless, fine needles, and had m.p. 212~213° and $[\alpha]_D^{19}$ $-22.70°$ (c, 0.488).

Anal. Found: C, 81.79; H, 11.29. Calcd. for $C_{32}H_{52}O_2$: C, 81.99; H, 11.18.

It gave a yellow color with tetranitromethane. The infrared spectrum of this Acetate A is shown in Fig. 1, g.

Deacetylation with 3% ethanolic KOH-solution of this acetate afforded Alcohol A, which, recrystallized several times from methanol, crystallized as colorless plates, and had m.p. 202~203°, and $[\alpha]_D^{19}-35.88°$ (c, 0.385).

Anal. Found: C, 84.62; H, 11.73. Calcd. for $C_{30}H_{50}O$: C, 84.44; H, 11.81.

Treatment with Sulfuric Acid and Glacial Acetic Acid of Ilexol Acetate—Formation of Acetate A. An amount of 200 mg of ilexol was suspended in a mixture of 5 ml of benzene, 40 ml of glacial acetic acid, and 1.5 ml of conc. H_2SO_4 (d, 1.84), and the resulting suspension was refluxed for 5 hrs. on a water-bath, after which the resulting reaction mixture was diluted with water, and taken up in ether. After thorough washing with water and subsequent drying with Na_2SO_4, removal of the solvent gave the residue, which was dissolved in petroleum ether, chromatographed on a column of alumina (2×6 cm), and eluted with the same solvent.

After removal of the solvent, the residue was recrystallized several times from acetone, giving prismatic needles, m.p. 206~207°. Recrystallizations several times from a mixture of chloroform and methanol (1:1) afforded pure acetate, which crystallized as colorless needles, and had m.p. and mixed m.p. 212~213° on admixture with the foregoing Acetate A.

Anal. Found: C, 82.11; H, 11.42. Calcd. for $C_{32}H_{52}O_2$: C, 81.99; H, 11.18.

The infrared spectrum of this Acetate A agreed with that of the foregoing Acetate A.

Deacetylation with 3% ethanolic KOH-solution of this Acetate A afforded Alcohol A, which, recrystallized several times from methanol, crystallized as colorless plates, and had m.p. and mixed m.p. 202~203° on admixture with the foregoing Alcohol A.

Treatment with Hydrochloric Acid and Glacial Acetic Acid of Isoilexol Aacetate—Formation of Acetate A. An amount of 200 mg of isoilexol acetate was suspended in a mixture of 35 ml of glacial acetic acid and 2 ml of hydrochloric acid (d, 1.19), and the resulting suspension was refluxed for 8 hrs. on a water-bath, after which the resulting reaction mixture was diluted with water, and taken up in ether. After thorough washing with water, and then dried with Na_2SO_4. Removal of the solvent gave the residue, which was dissolved in petroleum ether, chromatographed on a column of alumina (2×6 cm), and eluted with a mixture of petroleum ether and benzene (5:2). Removal of the solvent mixture gave the residue, which, recrystallized several times from a mixture of chloroform and methanol (1:1), gave pure acetate, which crystallized as colorless needles, and had m.p. and mixed m.p. 212~213° on admixture with the foregoing Acetate A.

Acknowledgements. The author's heartiest thanks are due to Prof. Dr. S. Iseda of this Laboratory, to Prof. Dr. Y. Oshima of Kyushu University, and to Prof. Dr. E. Sebe of Kumamoto University, for their helpful

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[6] K. Yagishita, This Bulletin, **21**, 160 (1957).

advice and encouragement throughout the course of this work.

He is also deeply indebted to Messrs. H. Kozuma and K. Kawasaki of Technical Department of Minamata Factory, Shin Nippon Chisso Hiryo Co., Minamata, Kumamoto, for their courtesy of measuring infrared spectra, to Dr. K. Uyeno of Dojin Pharmaceutical Laboratory, Kumamoto, for measuring ultraviolet spectra, and to Mr. E. Aoyagi of Technical Department of Oomuta Factory, Mitsui Kagaku Kogyo Co., Oomuta, Fukuoka, for carrying out elementary analyses.

[Bull. Agr. Chem. Soc. Japan, Vol. 22, No. 3, p. 136～142, 1958]

Studies on Metabolic Products of *Hypochnus sasakii* Shirai

Isolation of *p*-Hydroxyphenylacetic Acid and its Physiological Activity

By Yu-Shih Chen

Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo

Received December 21, 1957

A considerable amount of *p*-hydroxyphenylacetic acid was isolated from the culture filtrate of *Hypochnus sasakii*.

p-Hydroxyphenylacetic acid induced wilt of soybean plant and retarded the growth of rice seedlings at concentrations up to 1:20,000. The O_2 uptake in roots decreased in proportion to reduction of dry weight of rice seedlings.

Sheath blight (Mongare-byô) of rice plant, caused by *Hypochnus sasakii* Shirai (=*Pellicularia sasakii*, *Corticium sasakii*) is a fungal disease widely spread in the East Asia. In Japan, it is one of the most serious diseases of the rice plant, the annual loss in yield being estimated as 24,000～38,000 tons[1]. In severely affected fields, losses amounting to 30% in grain yield, and 20% in straw, have been reported[2].

Large greenish-grey lesions appear on the lower leaf sheathes and later spread to the leaf blades. If the environment is favorable for the growth of the fungus, it extends upwards, killing the upper leaves including the flag leaf.

The pathogene, *Hypochnus sasakii*, invades a wide range of plants, including soybean, corn, parsley, millet, the water-oat, many weeds and grasses which are grown around the rice fields, causing sheath blight and foliage rot.

The characteristic large greenish-grey lesion and its effect on the physiology of rice plant attracted the attention of the author and led to the assumption that the pathological effect may be involved with a certain toxic metabolic product of the pathogene, therefore, the author attempts were made to isolate the biologically active substance. By means of carbon adsorption, solvent extraction and purification with silica-gel partition chromatography, a physiological substance was obtained.

---

[1] National Institute of Agricultural Science, Section of Plant Pathology and Entomology, Tokyo, Japan (Prepared for the Fourth Session of the International Rice Commission, FAO, 1954): Insects and Diseases of Rice Plants in Japan p. 19 (1954).

[2] Y. Yokogi and M. Adachi, *Agriculture and Horticulture*, **27**, 701–702, 819–820 (1952).

This crystalline substance melts at 148–149°C, sublimes, and is readily soluble in cold water, hot water, ether, acetone, methanol, ethanol, butanol, ethylacetate and butylacetate, but insoluble in chloroform, benzene, ligroin and petroleum ether.

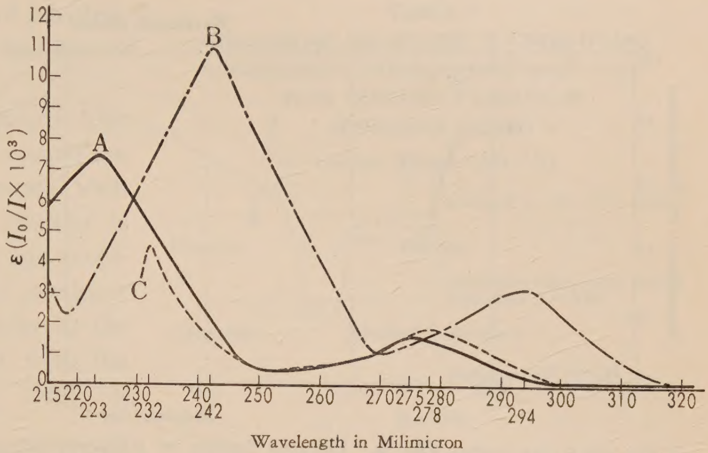


FIG. 2. U.V. Spectra of *p*-Hydroxyphenylacetic Acid.

A: Solvent, Distilled Water.
B: Absorption Spectrum of A at pH 12.
C: Solvent, Ethanol.

It induced wilt of soybean plant and at concentrations ranging from 1 : 2,000 to 1 : 20,000 reduced the length of leaves, especially, the dry weight of rice seedlings. The O_2 uptake in the top of rice seedlings hardly differed from the control at all concentrations, but the O_2 uptake in the roots was inhibited considerably at concentrations ranging from 1 : 2,000 to 1 : 100,000. The O_2 uptake in roots decreased in proportion to the reduction of dry weight of the rice seedlings.

From the results of elementary analysis and the Rast camphor test for molecular weight, it was confirmed that the molecular formular corresponds to $C_8H_8O_3$.

PK_a values for this substance are as shown in Fig. 1, $pk_1=4.3$, $pk_2=10.3$.

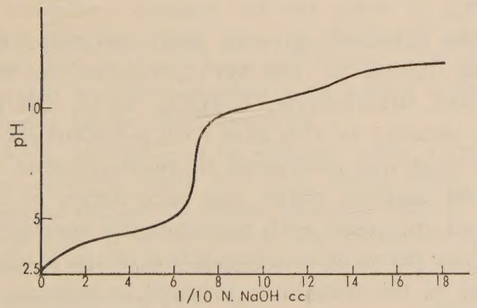


FIG. 1. Titration Curve of *p*-Hydroxyphenylacetic Acid.

The ultraviolet spectrum (Fig. 2) in distilled water showed absorption peaks at $\lambda_{\max.}$ 223mμ (ε 7500) and $\lambda_{\max.}$ 275 mμ (ε' 1600), while in ethanol it showed at $\lambda_{\max.}$ 232mμ (ε 4500) and $\lambda_{\max.}$ 278mμ (ε' 1800).

However, at the alkaline side they shifted to longer wave lengths and increased their molecular extinctions. The infrared spectrum as Nujol-mull (Fig. 3, 4) showed characteristic bands of –OH at 3240 and 1215, that of –COOH at 2700, 1700 and 1410, that of aromatic ring at 1610, 1600 and 1520, and that of aromatic 1 : 4 disubstitution at 790 cm^{-1}.

From the results of ultraviolet and infrared absorption spectra, existence of the phenolic hydroxy group was presumed.

In paper chromatography using *n*-butanol : acetic acid : water (4 : 1 : 5), benzene : acetic acid : water (2 : 1 : 1) or NH_4OH sat'd butanol as the developing solvents, R_F values were found to be 0.94, 0.85 and 0.20 respectively, using B. P. B and diazotized sulfanilic acid as detection reagents.

The color reactions showed positive for diazo, Millon, Potassium permanganate solution decolorization, bromine water decolorization and ferric chloride solution tests (dark

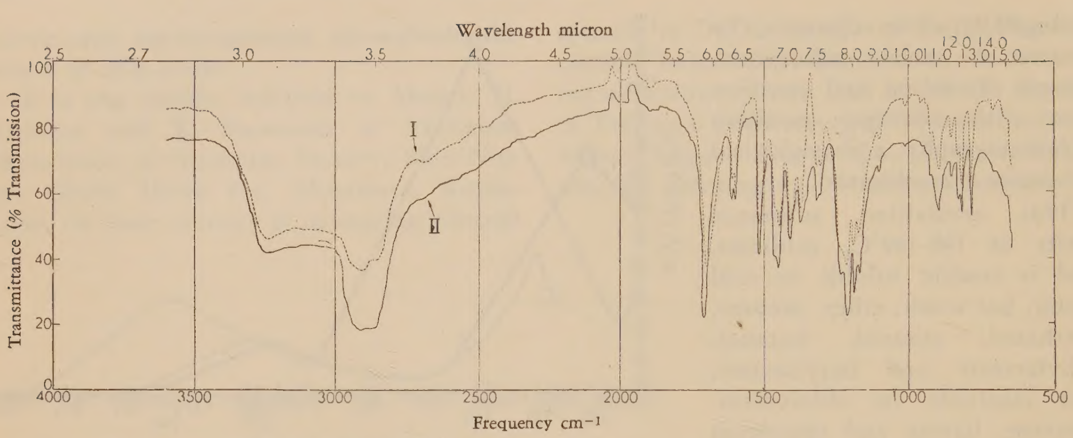


FIG. 3. Infrared Spectra of *p*-Hydroxyphenylacetic Acid.

I. Isolated compound from fungus. II. Synthetic compound.

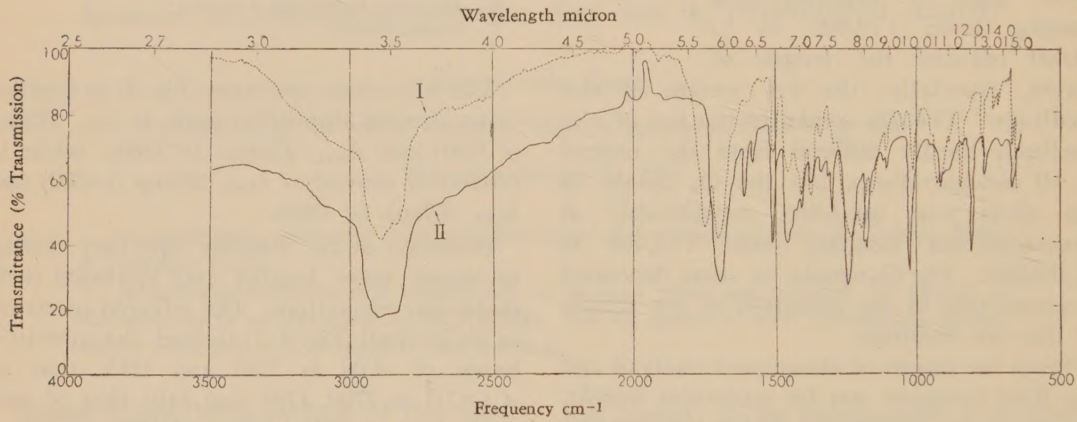


FIG. 4. Infrared Spectra of *p*-Hydroxyphenylacetic Acid Derivative.

I. *p*-Acetoxyphenylacetic acid. II. *p*-Methoxyphenylacetic acid.

yellow to pale brown), but negative for Fehling's, fuchsin and phloroglucinol-hydrochloric acid tests.

No nitrogen, halogen and sulfur were detected. By the Kuhn-Roth method, only 0.1 equivalent of C–CH_3 was detected indicating the absence of the C–CH_3 group in the molecule of original substance.

On reduction with Palladium-charcoal catalyser, no hydrogen absorption was confirmed.

It gave monomethyl-ether, $C_9H_{10}O_3$, m. p., 87°C, monoacetate, $C_{10}H_{10}O_4$, m. p., 108–109°C and *p*-bromophenacyl ester, $C_{16}H_{13}O_4Br$, m.p., 117°C.

This substance gave an acidic substance by alkali fusion at 250–280°C, soluble in aq. sodium bicarbonate, $C_7H_6O_3$, m. p., 214°C. The identity of this acid with *p*-hydroxybenzoic acid was confirmed by no depression of mixed melting point and accordance of infrared spectrum with an authentic specimen.

Upon permanganate oxidation of the methyl ether of the metabolic product, a substance, $C_8H_8O_3$, m.p., 178°C was obtained, which was proved to be identical with *p*-methoxybenzoic acid, prepared by the methylation of *p*-hydroxybenzoic acid by mixed fusion and accordance of infrared spectrum.

Taking the above mentioned results into account, it seemes likely that the crystalline substance of m.p., 148–149°C is identical with *p*-hydroxyphenylacetic acid, and identity is confirmed by mixed melting point and accordance of infrared spectrum of the synthetic *p*-hydroxyphenylacetic acid, henceforth, the identity of the metabolic product with the synthetic *p*-hydroxyphenylacetic acid is clearly established.

The author also proved that, *p*-hydroxyphenylacetic acid is contained in the rice plant attacked by sheath blight, by application of paperchromatography and ultraviolet spectrum.

EXPERIMENTAL

1. Isolation of the toxic product from cultured broth. *Hypochnus sasakii* used in this work was furnished by Mr. T. Ishie. It was cultured at 27–29°C for about 50 days in a modification of the Richard solution, i.e., sucrose 30 g, KNO_3 10 g, KH_2PO_4 5 g, $MgSO_4$ 2.5 g, peptone 2 g and H_2O 1*l*. Each 1,2*l*-aliquot of the medium was poured into a 3*l*-Fernbach flask.

The technique of bioassay was performed using soybean plant as the test plant. Firstly, a cutting of soybean seedlings was placed into the filtrate of the cultured broth and into an aliquot from each fraction in the process of isolation.

Secondly, a detached leaf was placed in a moist Petri dish, and drops from the filtrate of cultured broth and an aliquot from each fraction were placed on each needle-sticked points on the leaflets, to observe whether lesions will be produced.

From this filtrate of the cultured broth, a crystalline substance which acts as a wilting factor, was obtained through the process shown in Table I. The yield from 1*l* of the filtrate was 160 mg as in the form of the crude substance.

Mixed melting point test.

A mixture with an authentic *p*-hydroxyphenylacetic acid (m.p., 148–149°C) melted at 148–149°C.

TABLE I

PROCESS OF SEPARATING A CRYSTALLINE SUBSTANCE (*p*-Hydroxyphenylacetic Acid) FROM CULTURE FILTRATE OF *HYPOCHNUS SASAKII*

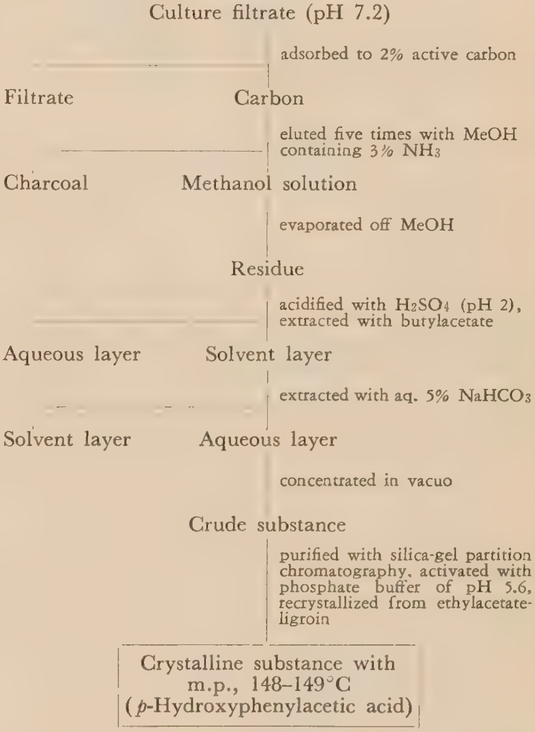

Culture filtrate (pH 7.2)
adsorbed to 2% active carbon
Filtrate
Carbon
eluted five times with MeOH containing 3% NH_3
Charcoal
Methanol solution
evaporated off MeOH
Residue
acidified with H_2SO_4 (pH 2), extracted with butylacetate
Aqueous layer
Solvent layer
extracted with aq. 5% $NaHCO_3$
Solvent layer
Aqueous layer
concentrated in vacuo
Crude substance
purified with silica-gel partition chromatography, activated with phosphate buffer of pH 5.6, recrystallized from ethylacetate-ligroin
Crystalline substance with m.p., 148–149°C (*p*-Hydroxyphenylacetic acid)

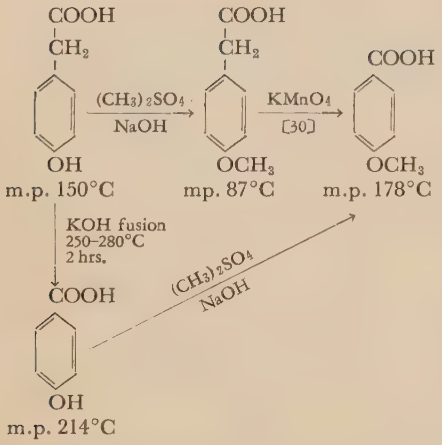
TABLE II

MOLECULAR FORMULAR OF METABOLIC PRODUCT AND THE MIXED MELTING POINT TEST WITH *p*-HYDROXYPHENYLACETIC ACID

Elementary analysis.		
Found	C% 62.78	H% 5.33
Calculated for $C_8H_8O_3$	C% 63.20	H% 5.20
Molecular weight, determined by Rast's method:	160	
Molecular weight, determined by titration method:	158.4	
Calculated Molecular weight for $C_8H_8O_3$:	152	

2. Preparation of derivatives, alkali fusion and permanganate oxidation. **a.** Monomethyl ether. The metabolic product (100 mg) was refluxed with 2 cc of dimethylsulfate and 15 cc of 5% sodium hydroxide solution for one hour.

The solution was cooled, filtered, acidified and extracted with ether, and recrystallized from ethylacetate-



ligroin: fine needles of *p*-methoxyphenylacetic acid (73 mg), $C_9H_{10}O_3$ (*Anal.* Calcd., C, 65.10, H, 6.09: C, 65.40, H, 6.20%), $OCH_3=19.01\%$ (*Anal.* Calcd., 18.87%), melting at 87°C was obtained.

b. Monoacetyl derivative. The metabolic product (150 mg), 5 cc of acetic anhydride and 10 cc of pyridine were mixed and kept in a closed flask at room temperature for 2 days. The mixture was evaporated to dryness, and the dry residue was purified by recrystallization from ethylacetate-ligroin, monoacetate (124 mg), $C_{10}H_{10}O_4$ (*Anal.* Calcd., C, 61.80, H, 5.10: Found, C, 61.62, H, 5.14%), $COCH_3=22.9\%$ (*Anal.* Calcd., 23.2%), melting at 108–109°C was obtained.

c. *p*-Bromophenacyl ester. The metabolic product (100 mg) was added to 2 cc of water in a small flask and neutrallized with N/10 sodium hydroxide solution. Two milliliters of alcohol and 100 mg of the phenacyl bromide were added, the mixture then refluxed for one hour. The solution was cooled and the precipitated ester was purified by recrystallization from alcohol, thus, *p*-bromophenacyl ester (46 mg), $C_{16}H_{13}O_4Br$ (*Anal.* Calcd., C, 55.01, H, 3.72, Br, 22.92: Found, C, 54.49, H, 4.22, Br, 22.87%), melting at 117°C was obtained.

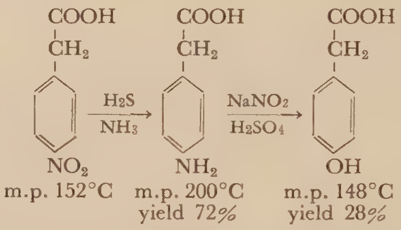
d. *p*-Methoxybenzoic acid. *p*-Hydroxybenzoic acid (200 mg) was refluxed with 2 cc of dimethylsulfate and 15 cc of 5% sodium hydroxide solution for one hour.

The solution was cooled, filtered, acidified and extracted with ether. Upon recrystallization from ethylacetate-ligroin, fine needles of *p*-methoxybenzoic acid (165 mg), $C_8H_8O_3$ (*Anal.* Calcd., C, 63.20, H, 5.20: Found, C, 63.28, H, 5.24%), melting at 117°C was obtained.

e. Alkali fusion. The metabolic product (150 mg) was fused with 3 g of potassium hydroxide and 250–280°C for 2 hours. The reaction mixture was added with distilled water, acidified with sulfuric acid and extracted with ether. The ether layer was extracted with 5% aqueous sodium bicarbonate solution and the latter acidified, then extracted with ether, and recrystallized from ethylacetate-ligroin: *p*-hydroxybenzoic acid (24 mg), $C_7H_6O_3$ (*Anal.* Calcd., C, 60.90, H, 4.30: Found, C, 60.96, H, 4.37%), melting at 214°C was obtained.

f. Permanganate oxidation. The methyl ether of the metabolic product (100 mg) was dissolved in 1 cc of 5% sodium hydroxide solution and 6 cc of water containing with 0.3 g of potassium permanganate was added under stirring dropwise, and the mixture heated at 50°C under reflux until the purple color of the permanganate disappeared. The mixture was cooled, filtered, acidified and extracted with ether, and recrystallized from ethylacetate-ligroin: fine needles of *p*-methoxybenzoic acid (67 mg), $C_8H_8O_3$ (*Anal.* Calcd., C, 63.20, H, 5.20: Found, C, 63.16, H, 5.28%), melting at 117°C was obtained.

3. Synthesis of *p*-hydroxyphenylacetic acid[3].



p-Nitrophenylacetic acid (20 g) was slowly added to 100 cc of 6N aq. ammonia with shaking. The mixture was placed in an ice bath, and saturated with hydrogen sulfide, then, the solution of ammonium *p*-aminophenylacetic acid was gently boiled until nearly all the excess hydrogen sulfide and ammonia had expelled. The deposited sulfur was filtered off, and 10 cc of glacial acetic acid rapidly stirred into the hot filtrate.

After the crude material was recrystallized from distilled water, a product (12.01g) which melted at 199–200°C, *p*-aminophenylacetic acid, was obtained.

A solution of the *p*-aminophenylacetic acid (6 g) in 50 cc of 6N sulfuric acid was cooled to 0°, treated with sodium nitrite (3 g) and the mixture kept at 0° for one hour with shaking, and then, heated at 40°. The mixture was cooled, filtered and extracted with ether, and recrystallized from ethylacetate-ligroin: *p*-hydroxyphenylacetic acid (1.68 g), melting at 148–149°C was obtained.

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3) G. Ross Robertson, Organic Synthesis Collective Volume 1, 52–53 (1948).

TABLE III

A COMPARISON OF THE LENGTH OF FIRST-LEAVES AND SECOND-LEAVES OF RICE SEEDLINGS TREATED WITH VARIOUS CONCENTRATIONS OF *p*-HYDROXYPHENYLACETIC ACID (Average of 10 Seedlings)

Concentration	Cont.	2.5×10^{-3}	5×10^{-3}	10×10^{-3}	20×10^{-3}	40×10^{-3}	100×10^{-3}	200×10^{-3}
Relative length of first-leaves (%)	100	74.9	96.0	101.0	100.4	107.3	110.8	115.4
Relative length of second-leaves (%)	100	61.7	76.9	86.4	92.5	102.7	106.9	109.3

TABLE IV

A COMPARISON OF DRY WEIGHTS OF RICE SEEDLINGS TREATED WITH VARIOUS CONCENTRATIONS OF *p*-HYDROXYPHENYLACETIC ACID (Average of 10 Seedlings)

Concentration	Cont.	2.5×10^{-3}	5×10^{-3}	10×10^{-3}	20×10^{-3}	40×10^{-3}	100×10^{-3}	200×10^{-3}
Relative weight (%)	100	52.6	68.4	78.9	88.7	95.4	98.6	102.6

4. Detection of *p*-hydroxyphenylacetic acid in the affected rice plant. Infected portions of the rice plant affected by *Hypochnus sasakii* were collected, chopped finely, extracted with 50% methanol solution, and the methanol was evaporated off. After acidification with dilute sulfuric acid, the residue was extracted with ether, subsequently by paper chromatography using *n*-butanol: acetic acid: water (4:1:5) system as the developing solvent. The area corresponding to R_F value 0.92 to 0.96 was cut off, extracted with acetone for about 24 hours and the acetone evaporated off. The ethanol solution of this residue showed absorption peaks at 233 mμ (ε 4600) and

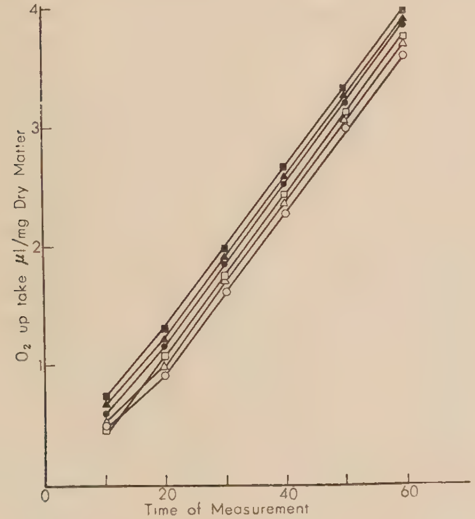

FIG. 5. Effect of the O_2 up taken on the Top of the Rice Seedlings and Various Concentrations of *p*-Hydroxyphenyl Acid.

—□— cont, —○— 2×10^{-3}, —△— 8×10^{-3}, —●— 32×10^{-3}, —▲— 100×10^{-3}, —■— 200×10^{-3}

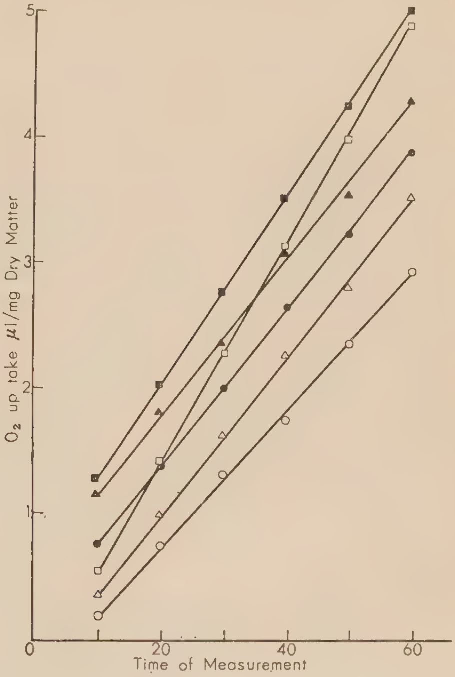

FIG. 6. Effect of the O_2 up take on the Roots of the Rice Seedlings and Various Concentrations of *p*-Hydroxyphenylacetic Acid.

—□— cont, —○— 2×10^{-3}, —△— 8×10^{-3}, —●— 32×10^{-3}, —▲— 100×10^{-3}, —■— 200×10^{-3}

278 mμ (ε' 1820). These results indicate that *p*-hydroxyphenylacetic acid exists in the infected portions of the rice plant attacked by *Hypochnus sasakii*.

5. Growth test of rice seedlings. Young seedlings of rice variety Nōrin 1, similar in height, were planted to a Petri dish covered with 1% agar, containing various concentrations of *p*-hydroxyphenylacetic acid, and kept in a glass container at 30° for 10 days, from September 12 to September 22, 1956, and the length of the first-leaves and second-leaves, and dry weight were measured, the results of which are shown in the following Table III and Table IV.

6. Measurement of O_2 uptake. The O_2 uptake was measured with Warburg's manometer (each compartment having the following contents).

Main Compartment: Five milli-liters of aq. *p*-hydroxyphenylacetic acid at concentrations ranging from 1:2,000 to 1:100,000.

Center Well: One milli-liter of 40% KOH solution.

The rice seedlings were steeped into aq. *p*-hydroxyphenylacetic acid at concentrations ranging from 1:2,000 to 1:100,000 and kept in a glass container at 30° for 24 hours, and then cut into two parts, one being the top of the seedlings and the other the root.

After the manometer stand came to a constant reading, aq. *p*-hydroxyphenylacetic acid and the test parts were mixed, and the vessel was shaken. The results are shown in Fig. 5 and Fig. 6.

Acknowledgement. The author wishes to express his sincere thanks to Prof. Y. Sumiki and Prof. H. Asuyama for their kind guidance throughout this work. Indebts are due to the Central Research Institute of Japan Monopoly Corporation for measurements of infrared spectra and also to Mr. T. Ishie for furnishing the pathogene of *Hypochnus sasakii*.

[Bull. Agr. Chem. Soc. Japan, Vol. 22, No. 3, p. 143~153, 1958]

Microbiological Studies of *Coli-aerogenes* Bacteria*

Part V. A Cyclic Mechanism of Aerobic Carbon-Metabolism

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Received December 23, 1957

α-Ketoglutarate was obtained in a very small amount by the oxidative fermentation of acetate with either a growing culture or the washed cells of *Escherichia coli*. This microorganism was also observed to accumulate a considerable amount of α-ketoglutarate as the oxidation-product of C_4-dicarboxylic acids such as succinate, fumarate, malate and oxalacetate. The addition of acetate to the reaction mixtures containing either C_3- or C_4-acids brought about an increase in the yield of α-ketoglutarate. The bacteria of *coli-aerogenes* revealed an ability of oxidizing tricarboxylic acids under suitable conditions, but there was no noticeable production of α-ketoglutarate. The formation of glyoxylate was observed to occur during the degradation of citrate by the bacteria of *coli-aerogenes*. Finally, a cyclic mechanism of aerobic carbon-metabolism in the bacteria was propounded and discussed.

INTRODUCTION

Although a large number of investigation have been performed, the role of the Krebs cycle in bacterial respiration has yet not been elucidated.

As regards oxidations of a member of the tricarboxylic acid cycle by *Escherichia coli*, Ajl et al. suggested from their earlier experiments with a simultaneous adaptation1) or labeled substrate2),3) that the Krebs cycle could not play an important role in the respiration of the bacterium. However, from their further investigations they have recently concluded that *E. coli* not only possesses all the enzymes which belong to the tricarboxylic acid cycle but it also oxidizes acetate by way of this cycle. In this case, the following facts constitute strong evidence in favour of their assumption.

(A) Incorporation of significant amounts of labeled acetate carbon into all the members of the Krebs cycle2),4).

(B) Occurrence of the condensing enzyme (enzyme synthesizing citrate from acetate and oxalacetate)5), isocitric dehydrogenase6), aconitase6) and α-ketoglutaric oxidase7).

On the other hand, it has been demonstrated by some investigators that *E. coli* splits citrate into C_2– and C_4–units6),8),9),10),11),12). In the case of the bacteria of *aerogenes* group, Ajl et al. have shown that citrate behaves like an intermediate in the respiration of citrate-grown cells but not in that of acetate-grown cells and it has been supposed by them that the acetate-grown cells oxidize acetate by way of Thunberg-Knoop or some other types of condensation, whereas, the citrate-grown cells appear to possess the tricarboxylic acid cycle as a mechanism for the oxidation of acetate13). Thus, these results indicate that the oxidation of acetate by the bacteria occurs by different mechanism, depending on the kind of source of carbon employed for their growth.

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* A part of this paper was read before the annual meeting of the Japanese Biochemical Society (November, 1955) and the Agricultural Chemical Society of Japan (March, 1956).

1) S. J. Ajl, *J. Bacteriol.*, **59**, 499 (1950).

2) S. J. Ajl and M. D. Kamen, *J. Biol. Chem.*, **189**, 845 (1951).

3) S. J. Ajl, *J. Gen. Physiol.*, **34**, 785 (1951).

4) S. J. Ajl and D. T. O. Wong, *Arch. Biochem. and Biophys.*, **54**, 474 (1955).

5) G. D. Novelli and F. Lipmann, *J. Biol. Chem.*, **182**, 213 (1950).

6) R. W. Wheat and S. J. Ajl, *Arch. Biochem. and Biophys.*, **49**, 7 (1954).

7) S. J. Ajl and C. H. Werkman, *Proc. Natl. Acad. Sci. U. S.*, **34**, 491 (1948).

8) M. Grunberg-Mango and I. C. Gunsalus, *Bact. Proc.*, P. 73 (1953).

9) R. W. Wheat and S. J. Ajl, *J. Biol. Chem.*, **217**, 897 (1955).

10) R. W. Wheat, D. T. O. Wong and S. J. Ajl, *J. Bacteriol.*, **68**, 19 (1954).

11) R. W. Wheat and S. J. Ajl, *J. Biol. Chem.*, **217**, 909 (1955).

12) D. T. O. Wong and S. J. Ajl, *Nature*, **176**, 970 (1955).

Recently, a number of studies on the bacterial formation of α-ketoglutarate have been carried out and the idea that it is produced by way of the tricarboxylic acid cycle has frequently been entertained14,15,16,17).

More recently, another idea has however been introduced by Weimberg et al. They have investigated the oxidation of L-arabinose by *Pseudomonas saccharophila* and found that α-ketoglutarate is produced from L-arabonate and L-arabinose by the action of this microorganism18). While, Koepsell et al. previously investigated the oxidation by a growing culture of *Pseudomonas fluorescens* of C_6-compounds such as glucose, gluconate, 2-ketogluconate and it was suggested by them that C_6-compounds would first be converted into pyruvate, and this pyruvate might condense with fragments such as C_2- or C_3-compounds being probably produced from C_6-compounds, to form α-ketoglutarate19,20).

In the previous paper the authors have investigated the oxidative fermentations of glucose, pyruvate and lactate by the bacteria of *coli-aerogenes* types and have revealed the fact that these bacteria induce α-ketoglutaric acid-fermentation21,22,23,24). The present investigations have been undertaken with the object of throwing some light on the mechanism of the aerobic metabolism of the bacteria.

EXPERIMENTAL AND RESULTS

Microorganisms. Two strains of the bacteria, *Escherichia coli* (G-2), *Aerobacter aerogenes* (B-2), were used through the experiments.

Analytical Methods. Ketonic acids were identified by paper-partition chromatography of the 2,4-dinitrophenylhydrazones after the method of Cavallini et al.25) and determined by a modified method of Friedemann and Haugen26). Acetic and citric acids were estimated by the methods of Friedemann27) and Kuratomi et al.28), respectively.

Detection of C_4-dicarboxylic acid such as succinate was carried out paper-chromatographically by the method of Aso et al.29)

Production of α-Ketoglutarate from Acetate. In the previous papers23,24), it has been demonstrated by the authors that pyruvate is converted into α-ketoglutarate by oxidative fermentation with the bacteria of *coli-aerogenes* types. It is, however, a well known fact that by action of the bacteria, pyruvate is oxidized to acetate along with evolution of carbon dioxide. Thus, it is of interest to ascertain whether or not α-ketoglutarate would be produced during the oxidation of acetate by the bacteria.

In the present paper, experiments of a growing culture of *E. coli* were at first carried out with a medium (pH: 7.0) containing 3.0% Na-acetate, 0.05% $(NH_4)_2HPO_4$, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, and 0.5% NaCl. The bouillon culture of *E. coli* obtained by 2 days' incubation at 37°C under static conditions was used as an inoculum. From the results shown in Table I, in which fermentations were carried out for 6 days at 28°C under shaking or static conditions, it was

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13) S. J. Ajl and D. T. O. Wong, *J. Bacteriol.*, **61**, 379 (1951).

14) S. Dagley, M. E. Fewster and F. C. Happold, *J. Bacteriol.*, **63**, 327 (1952).

15) J. J. R. Campbell and F. N. Stokes, *J. Biol. Chem.*, **190**, 853 (1951).

16) M. Kogut and E. P. Podoski, *Biochem. J.*, **55**, 800 (1954).

17) E. Masuo, Y. Nozaki, Y. Wakisaka and K. Yoshida, *Symposia on Enzyme Chemistry*, **8**, 105 (1953).

18) R. Weimberg and M. Doudoroff, *J. Biol. Chem.*, **217**, 607 (1955).

19) H. J. Koepsell, *J. Biol. Chem.*, **186**, 743 (1950).

20) H. J. Koepsell, F. H. Stodola and E. S. Sharpe, *J. Am. Chem. Soc.*, **74**, 5142 (1952).

21) H. Katagiri, T. Tochikura and K. Imai, This Bulletin, **21**, 210 (1957).

22) H. Katagiri, T. Tochikura and K. Imai, This Bulletin, **21**, 215 (1957).

23) H. Katagiri, T. Tochikura and K. Imai, This Bulletin, **21**, 346 (1957).

24) H. Katagiri and T. Tochikura, This Bulletin, **21**, 351 (1957).

25) D. Cavallini, N. Frontali and G. Toschi, *Nature*, **163**, 568 (1949).

26) T. E. Friedemann and G. E. Haugen, *J. Biol. Chem.*, **147**, 415 (1943).

27) T. E. Friedemann, *J. Biol. Chem.*, **123**, 161 (1938).

28) K. Kuratomi and N. Hosoya, *J. Japan. Biochem. Soc.*, **27**, 73 (1955).

29) K. Aso et al, *J. Fermentation Technol.*, **29**, 167 (1951).

TABLE I

PRODUCTION OF α-KETOGLUTARATE FROM ACETATE BY GROWING CULTURE OF *E. COLI*

Cultural method	Shaking culture	Static culture
Consumption of acetate (%)	90.9	17.3
α-Ketoglutarate produced (mM/dl)	0.328	0
Pyruvate produced (mM/dl)	0.125	Trace

found that under aerobic conditions, acetate was considerably oxidized and production of α-ketoglutarate took place, whereas, under static conditions the consumption of acetate was feeble. The experimental results obtained by a growing culture were further ascertained with the washed cells of *E. coli* in the following manner. After being grown with a shaker at 30°C for 2 days in a medium containing 2% glucose, 0.1% $(NH_4)_2HPO_4$, 0.3% $(NH_4)_2SO_4$, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.2% NaCl, and 1% $CaCO_3$, the washed cells of *E. coli* were prepared in the same manner as described in the previous paper.

To the washed cells (100–280 mg as dried matter) were added (1) 5–2.5 mM phosphate buffer (pH: 6.2), (2) 4–2 mM Na-acetate, (3) 40 μM $MgSO_4$ and (4) water to make the total volume 20–30 ml. The mixture was placed on a shaker and incubated for 14 hours at 30°C. Table II shows that the washed cells of *E. coli* grown on a glucose-medium are capable of producing α-ketoglutarate from acetate as a sole source of carbon under aerobic conditions, the maximal yield being about 0.11 mole per one mole of acetate consumed.

TABLE II

PRODUCTION OF α-KETOGLUTARATE FROM ACETATE BY WASHED CELLS OF *E. COLI* GROWN AEROBICALLY ON GLUCOSE MEDIUM

Exp. No.	I	II	III
Phosphate buffer (mM)	2.5	2.5	5.0
$MgSO_4$ (μM)	40	40	40
Bacterial cells (mg)	100	100	280
Total volume (ml)	20	20	30
Acetate initial (mM)	4.000	2.000	4.000
Acetate consumed (mM)	1.100	1.810	3.790
α-Ketoglutarate produced (mM)	0.079	0.160	0.413
(Molar yield)	0.07	0.09	0.11

In this case, however, it should be noted that the yield is extremely dependent upon the experimental conditions, since only a trace amount of α-ketoglutarate can be detected in the case of other experiments which are not described in Table II.

Dagley et al. have found that the oxidative fermentation of acetate by *Aerobacter aerogenes* gives rise to a very small amount of pyruvate under suitable conditions[30]. On the other hand, Ajl et al. have shown that the simultaneous oxidation by *E. coli* of labeled acetate and unlabeled C_4-dicarboxylic acids leads to incorporation of labeled carbon into these C_4-acids[2]. Thus, it may be suggested that the formation of α-ketoglutarate from acetate occurs in the following manner.

(1) A formation of succinate from acetate by way of either Thunberg-Knoop-condensation[31,32,33] or glyoxylate cycle[34,35].

(2) The formation of pyruvate from succinate via fumarate, malate and oxalacetate.

(3) The formation of α-ketoglutarate by way of a certain condensation reaction[24] in which pyruvate is concerned.

Production of α-Ketoglutarate from C_4-Dicarboxylic Acids. The experimental results mentioned in previous papers[23,24], in which α-ketoglutarate was obtained in a high yield from C_3-compounds as pyruvate and lactate, led the authors to enquire whether this C_5-acid would be produced from dicarboxylic acids including succinate, fumarate, malate, and oxalacetate.

Experiments were carried out with the washed cells of *E. coli* grown aerobically on a glucose medium in the same manner as in those experiments shown in Table II. From the results given in Table III, the accumulation of α-ketoglutarate was found to occur when the washed cells were incubated on a shaker at 30°C for 12–15 hours with the media containing phosphate buffer (pH: 6.2), $MgSO_4$, and various dicarboxylic acids.

In this case, the yield of α-ketoglutarate with dicarboxylic acids appeared not to be higher than that with pyruvate. Furthermore, it was ascertained that at an early stage in the oxidation of dicarboxylic acids, there was an accumulation of a trace amount

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[30] S. Dagley, E. A. Dawes and G. A. Morrison, *J. Gen. Microbiol.*, **5**, 508 (1951).

[31] T. Thunberg, *Skand. Arch. Physiol.*, **40**, 1 (1920).

[32] F. Knoop, *Klin. Wochschr.*, **2**, 60 (1923).

[33] G. R. Seaman and M. D. Naschke, *J. Biol. Chem.*, **217**, 1 (1955).

[34] D. T. O. Wong and S. J. Ajl, *J. Am. Chem. Soc.*, **78**, 3230 (1956).

[35] H. L. Kornberg and H. A. Krebs, *Nature*, **179**, 988 (1957).

TABLE III

PRODUCTION OF α-KETOGLUTARATE FROM C_4-DICARBOXYLIC ACIDS BY WASHED CELLS OF *E. COLI* GROWN AEROBICALLY ON GLUCOSE MEDIUM

Substrate	Oxalacetate	Fumarate	L-Malate	Succinate
(mM)	1.510	3.100	0.400	3.000
Phosphate buffer (mM)	5.0	5.0	1.0	5.0
$MgSO_4$ (μM)	100	40	10	100
Bacterial cells (mg)	185	120	35	193
Total volume (ml)	50	50	5.5	40
Time of incubation (hours)	13	12	15	12
α-Ketoglutarate produced (mM)	0.142	0.152	0.044	0.693
(Molar yield)	0.09	0.05	0.11	0.23

of pyruvate which subsequently disappeared. From this observation it has been pointed out that the oxidation of C_4-dicarboxylic acids with the production of α-ketoglutarate proceed via pyruvate. Dagley et al. have previously investigated the oxidation by *Aerobacter aerogenes* of dicarboxylic acids such as succinate, fumarate and malate and observed that pyruvate is accumulated in the aerated cultures containing those C_4-acids by action of the bacterium[30]. On the other hand, *coli-aerogenes* bacteria are well known to metabolize dicarboxylic acid to CO_2 and H_2O (complete respiration) or cell materials (oxidative assimilation). Accordingly, it has been recognized that dicarboxylic acids are metabolized to CO_2 and H_2O or cell-materials by way of a definite reaction in which pyruvate or α-ketoglutarate is concerned.

Effect of Acetate on the Production of α-Ketoglutarate. In view of the fact that C_3- or C_4-acids are a potent precursor in the bacterial production of α-ketoglutarate and are also oxidized via acetate, it was of interest to learn whether the yield of α-ketoglutarate from these acids would result in an increase in presence of externally added acetate. The reaction mixtures prepared in the same manner as already mentioned, which contained phosphate buffer, $MgSO_4$, Na-salts of C_3- or C_4-acids, and the washed cells of *E. coli* grown on a glucose-medium, were incubated at 30°C on a shaker either in presence or absence of externally added acetate.

From the results shown in Table IV–VI, it has been found that the oxidation of C_3- or C_4-acids by the washed cells of the bacterium give rise to a much

TABLE IV

INFLUENCE OF ADDITION OF ACETATE ON PRODUCTION OF α-KETOGLUTARATE FROM PYRUVATE

Bacterial cells (mg)	Time of incubation (hours)	Pyruvate initial (mM)	Pyruvate consumed (mM)	Acetate initial (mM)	Acetate consumed (mM)	α-Ketoglutarate produced (mM)
138	14	4.000	4.000	0		1.110
138	14	4.000	4.000	2.000	2.000	1.422
138	14	0		2.000	2.000	Trace
245	15	2.000	2.000	0		0.400
245	15	2.000	2.000	2.000	2.000	0.795
245	15	0		2.000	2.000	Trace
138	14	2.000	2.000	0		0.287
138	14	2.000	2.000	2.000	2.000	0.640
145	15	4.000	4.000	0		1.160
145	15	4.000	4.000	2.000	2.000	1.450
435	15	4.000	4.000	0		0.493
435	15	4.000	4.000	2.000	2.000	0.750

Each vessel contained 5mM phosphate buffer (pH: 6.0), 100 μM $MgSO_4$, stated amounts of washed cells of *E. coli*, Na-pyruvate and Na-acetate.; total volume 40 ml; temp. 30°C.

larger amount of α-Ketoglutarate in presence of externally added acetate than in its absence. With regard to the effect of acetate on the production of α-ketoglutarate, other experiments with the washed cells of *E. coli* were carried out in the following manner.

(1) Na-acetate was added to the mixtures containing phosphate buffer, $MgSO_4$ and C_3- or C_4-acids before incubation and then fermentations by the washed cells of *E. coli* were carried out at 30°C on a shaker.

(2) At first, the mixtures containing the washed cells, C_3- or C_4-acids and the other mineral matters were incubated on a shaker in the absence of externally added acetate for 8 hours at 30°C and thereafter, Na-acetate was added to the mixtures and fermentations were continued.

TABLE V

INFLUENCE OF ADDITION OF ACETATE ON PRODUCTION OF α-KETOGLUTARATE FROM L-LACTATE

Addition of L-Lactate (mM)	Addition of acetate (mM)	α-Ketoglutarate produced (mM)
2.500	2.000	0.616
2.500	0	0.200

Each vessel contained 5 mM phosphate buffer (pH: 6.2), 100 µM $MgSO_4$, 110 mg washed cells of *E. coli*, stated amounts of Na-L-lactate and Na-acetate and water to 40 ml. Incubation for 15 hours at 30°C.

The results are shown in Table VII from which it has been observed that greater amounts of α-ketoglutarate are obtained when acetate is added before incubation than those obtained when it is added after 8 hours' incubation. Thus, both acetate (acetyl-CoA) and pyruvate appear to be potent precursors in the formation of α-ketoglutarate.

Oxidation of Tricarboxylic Acids by *Coli-Aerogenes* Bacteria. As regards the production of α-ketoglutarate by the microorganisms, Dagley et al.

TABLE VI

INFLUENCE OF ADDITION OF ACETATE ON PRODUCTION OF α-KETOGLUTARATE FROM DICARBOXYLIC ACIDS

Exp. No.	Bacterial cells (mg)	Addition of dicarboxylic acids (mM)		Addition of acetate (mM)	α-Ketoglutarate produced (mM)
I—a	193	Succinate	2.000	2.000	0.898
I—b	193	Succinate	3.000	0	0.693
I—c	193	Succinate	0	2.000	Trace
II—a	185	Oxalacetate	1.500	2.000	0.500
II—b	185	Oxalacetate	1.500	0	0.142
III—a	120	Fumarate	1.550	2.000	0.540
III—b	120	Fumarate	1.550	0	0.233
IV—a	210	L-Malate	2.800	2.800	0.925
IV—b	210	L-Malate	2.800	0	0.310

Each vessel contained 5 mM phosphate buffer (pH: 6.2), 100 µM $MgSO_4$, stated amounts of Na-salts of dicarboxylic and acetic acids, washed cells of *E. coli* and water to 50 ml. Incubation for 12 hours at 30°C.

TABLE VII

INFLUENCE OF ADDITION-TIME OF ACETATE ON PRODUCTION OF α-KETOGLUTARATE FROM C_3- OR C_4-ACIDS

Bacterial cells (mg)	Addition of C_3- or C_4-acids (mM)		Addition of acetate (mM)	α-Ketoglutarate produced (mM)
138	Pyruvate	3.000	3.000 (added before incubation)	1.160
138	Pyruvate	3.000	3.000 (added after 8 hours' incubation)	0.826
200	L-Lactate	2.500	3.000 (added before incubation)	0.450
200	L-Lactate	2.500	3.000 (added after 8 hours' incubation)	0.200
133	Succinate	2.000	2.000 (added before incubation)	0.653
133	Succinate	2.000	2.000 (added after 8 hours' incubation)	0.358

Each vessel contained 5 mM phosphate buffer (pH: 6.2), 100 µM $MgSO_4$, stated amounts of washed cells of *E. coli*, Na-pyruvate, Na-lactate, Na-succinate and Na-acetate; total volume 40-60 ml. Incubation for 21 hours at 30°C.

TABLE VIII

PRODUCTION OF α-KETOGLUTARATE FROM PYRUVIC AND TRICARBOXYLIC ACIDS BY WASHED CELLS OF *A. AEROGENES*

Exp. No.		I	II	III	IV	V	VI	VII
Sources of carbon with which *A. aerogenes* was grown		Citrate	Citrate	Citrate	Citrate	Citrate	Citrate	Glucose
Substrates against which washed cells were tested		Citrate	Citrate	*cis*-Aconitate	iso-Citrate	Pyruvate	Pyruvate	Pyruvate
Bacterial cells (mg)		155	165	155	140	100	100	150
Total volume (ml)		40	40	40	30	30	40	40
Substrates	initial (mM)	0.700	1.068	0.690	1.000	4.000	4.000	4.000
	consumed (mM)	0.700	1.068			4.000	4.000	4.000
α-Ketoglutarate produced (mM)		0.099	0.049	Trace	0	0.654	0.800	0.640
(Molar yield)		0.141	0.046			0.164	0.200	0.160

who investigated the oxidation of succinate by *Vibrio* 01, suggested that the formation of α-ketoglutarate might occur either by a reductive carboxylation of succinate, that is to say, a reversal of α-ketoglutaric-oxidase, or via the tricarboxylic acid cycle[14].

A similar suggestion was proposed by Kogut et al., who investigated the production of α-ketoglutarate from succinate by the action of Pseudomonas fluorescens[16].

The authors' attention has been directed to the tricarboxylic acids from which α-ketoglutarate might be supposed to be formed, according to the Krebs cycle.

Experiments were at first carried out with the washed cells of *Aerobacter aerogenes* grown at 30°C for 2 days on a shaker in a medium (pH: 7.2) containing 3% Na-citrate, 0.25% peptone, 0.1% $(NH_4)_2HPO_4$, 0.25% $(NH_4)_2SO_4$, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$ and 0.2% NaCl. In this case, experiments with the washed cells of the bacterium grown aerobically on the same 2% glucose-medium as previously mentioned, were performed for the sake of comparison. The reaction mixtures (30–40 ml) contained 5–10mM phosphate buffer (pH: 6.0), 100 μM $MgSO_4$, requisite amounts of various substrates (Na-salts) and of washed cells of *A. aerogenes* as will be shown in Table VIII, and incubations were carried out on a shaker at 30°C for 10–15 hours.

The experimental results given in Table VIII may be summarized as follows.

(1) α-Ketoglutarate was obtained in the product of oxidative fermentation of citrate by the washed cells of *A. aerogenes* grown on a citrate-medium. According to the Krebs cycle, one mole of citrate yields one mole of α-ketoglutarate. However, it will be shown in Table VIII that only about 14–5% of the theoretical amount of α-ketoglutarate could be obtained. Moreover in the case of the other experiments which are not shown in Table VIII, the oxidation of citrate resulted in yielding only a trace amount of α-ketoglutarate.

(2) There was scarcely any production of α-ketoglutarate with isocitrate or *cis*-aconitate to be observed.

(3) The oxidation of pyruvate was ascertained to give rise to a large amount of α-ketoglutarate not only by the bacterial cells obtained from a glucose-medium, but also by those trained to citrate, the yields being about 0.4–0.5 mole per two moles of pyruvate oxidized. In other words, the yields with pyruvate amount to 40–50% of the theoretical value according to a certain "pyruvate-acetate reaction."

It has been recognized that pyruvate is by far more potent in the production of α-ketoglutarate than tricarboxylic acid.

Thus, these results appear to exclude a conventional tricarboxylic acid cycle as the major reaction producing α-ketoglutarate in *A. aerogenes*.

The idea that the major oxidation of tricarboxylic acid by *A. aerogenes* might occur by way of a reaction not involving α-ketoglutarate as the direct intermediate is further supported by the following experiments with a Warburg-technique. The content of manometer cup was as follows: in the side arm 7–15 mg (dry-weight) washed or dried cells of *A. aerogenes* grown with citrate, in the main cup 150–300 μM phosphate buffer (pH: 6.0), 4 μM $MgSO_4$ and those amounts of Na-citrate or Na-*cis*-aconitate as indicated, in the centre well 0.2 ml 15% KOH; total volume 2.5 ml. Incubations were carried out at 30°C for 2–7 hours in air. From the results shown in Table IX, it can be observed that citrate or *cis*-aconitate is largely oxidized, by *A. aerogenes* grown on citrate, with the evolution of carbon

TABLE IX

OXIDATION OF TRICARBOXYLIC ACIDS BY *A. AEROGENES* GROWN ON CITRATE MEDIUM

Bacterium	Substrates (μM)	O_2 absorbed (μM)	CO_2 evolved (μM)	Ketonic acid formed (as α-ketoglutarate) (μM)	(Molar yield)
	Citrate				
Washed cells	10.0	29.4	36.0	0.98	0.098
	5.0	15.0		Trace	
	cis-Aconitate				
Washed cells	40.0	66.8		2.12	0.053
	20.0	43.0		1.27	0.064
	Citrate				
Dried cells	2.5	11.0		0	

dioxide and with the uptake of oxygen, but there is not a remarkable production of α-ketoglutarate.

With regard to the behaviour of *E. coli* towards citrate, it has been shown by Lara et al.[36] and Ajl et al.[4] that the dried cells of the organism are capable of oxidizing citrate, whereas, the resting cells can not metabolize it.

This observation was again ascertained by the authors in the following experiments with dried-, dry ice treated-, ground- and intact-cells.

(1) Experiment with the ground-cells: Washed cells of *E. coli* grown on 2% glucose-medium were ground with powdered glass (3 times the cell-weight) at 0°C for 30 minutes and the glass was then removed by a centrifuge. The reaction mixture contained 200 μM phosphate buffer (pH: 6.7) 4 μM $MgSO_4$, 25 μM Na-citrate, 14 mg (as dry weight) bacterial cells, in the centre well 0.2 ml 15% KOH; total volume 2.5 ml. Oxygen uptake: about 15 μM after 155 minutes' incubation at 37°C in air. α-Ketoglutarate formed: negligible.

(2) Experiment with the dry ice-treated cells: The dry ice-treated cells were obtained by freezing and thawing procedures as previously reported[13]. The reaction mixture contained 300 μM phosphate buffer (pH: 6.0), 4 μM $MgSO_4$, 20 μM Na-citrate, 14 mg (as dry weight) bacterial cells, in the centre well 0.2 ml 15% KOH; total volume 2.5 ml. Oxygen uptake: about 6 μM after 245 minutes' incubation at 30°C in air. α-Ketoglutarate formed: negligible.

(3) Experiment with the dried-cells: The washed cells were dried by an electric fan at room temperature for 5 hours. The reaction mixture contained 200 μM phosphate buffer (pH: 6.4), 4 μM $MgSO_4$, 20 μM Na-citrate, 20 mg dried cells, in the centre well 0.2 ml 15% KOH; total volume 2.5 ml. Oxygen uptake: about 9 μM after 195 minutes' incubation at 37°C in air. α-Ketoglutarate formed: negligible.

(4) Untreated resting cells were observed to be incapable of oxidizing citrate: From these experiments, it is apparent that the oxidation of citrate actually occurs by action of the treated cells such as dried- or ground-cells of *E. coli* grown on a glucose-medium, but there is not any formation of α-ketoglutarate to be observed. The oxidation of citrate by the treated cells was moreover proved to proceed much slower than that of C_3- or C_4-acids.

These observations were also ascertained by macro-experiments conducted with the treated cells of *E. coli*, which were prepared in the following manner: (1) *E. coli* grown on a medium containing 2% glucose, 1% Na-citrate and other mineral matters, was harvested and then dried; (2) the bacterium grown on a glucose medium was frozen and ground with dry ice powder. These treated cells (100–500 mg as dry weight) were added to the media (40 ml) containing 5 mM phosphate buffer (pH: 6.0), 100 μM $MgSO_4$, 2–0.5 mM Na-citrate or Na-*cis*-aconitate and then incubated on a shaker at 30°C for 10–14 hours. After incubation, the analysis of α-ketoglutarate was performed. Results of the experiments revealed that the production of α-ketoglutarate was negligible even under the conditions using a large quantity of the treated cells and the substrates.

Recently, it has been demonstrated in many investigations that at least two mechanism exist, by which tricarboxylic acids are broken down anaerobically to form C_2- and C_4-fragments. One is concerned in the formation of acetate and oxalacetate[37~44], and

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[36] F. J. S. Lara and J. L. Stokes, *J. Bacteriol.*, **63**, 415 (1952).

[37] M. Deffner, *Ann.*, **536**, 44 (1938).

[38] M. Deffner and W. Franke, *Ann.*, **541**, 85 (1939).

[39] K. Sakaguchi and S. Tada, *Zent. f. Bakt. Parasit. u. Infect II Abteil, Bd.* 101 (1940).

[40] S. Tada, *J. Agr. Chem. Soc. Japan*, **16**, 663, 675 (1940); **17**, 569 (1941).

[41] S. Dagley and E. A. Dawes, *Nature*, **172**, 345 (1953).

[42] D. C. Gillespie and I. C. Gunsalus, *Bact. Proc.*, p. 80 (1953)

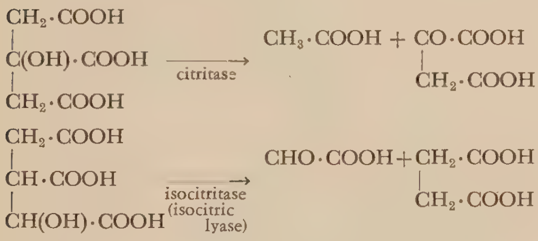
[43] H. Takahashi, *Symposia on Enzyme Chemistry*, **9**, 64 (1954).

[44] H. Takahashi and S. Sugama, *Symposia on Enzyme Chemistry*, **10**, 49 (1954).

TABLE X
FORMATION OF GLYOXYLATE FROM CITRATE BY DRIED CELLS OF BACTERIA OF *COLI-AEROGENES*

Kind of bacteria		*A. aerogenes* grown aerobically on citrate medium	*E. coli* grown aerobically on glucose medium
Addition of citrate (mM)		2.000	1.000
Addition of semicarbazide (mM)		4.5	4.5
Dried cells (mg)		225	1000 Cells were ground for 20 min. at 0°C with powdered glass
Reaction mixture	(initial pH)	6.5	7.4
	(ml)	50	50
Time of incubation under aerobic conditions (hours)		12	5
Glyoxylate formed (μM)		50	67

the other causes the occurrence of glyoxylate and succinate[12,45~49] as follows:



$$\begin{array}{l}CH_2\cdot COOH\\ |\\ C(OH)\cdot COOH\\ |\\ CH_2\cdot COOH\end{array} \xrightarrow[\text{citritase}]{} CH_3\cdot COOH + \begin{array}{l}CO\cdot COOH\\ |\\ CH_2\cdot COOH\end{array}$$

$$\begin{array}{l}CH_2\cdot COOH\\ |\\ CH\cdot COOH\\ |\\ CH(OH)\cdot COOH\end{array} \xrightarrow[\substack{\text{isocitritase}\\ \text{(isocitric}\\ \text{lyase)}}]{} CHO\cdot COOH + \begin{array}{l}CH_2\cdot COOH\\ |\\ CH_2\cdot COOH\end{array}$$

In order to gain an insight into the mechanism of the oxidation of tricarboxylic acids by *coli-aerogenes*, the following experiments with a trapping agent were instituted. Either the dried cells (225 mg) of *A. aerogenes* trained to citrate or those (1000 mg) of *E. coli* grown on a glucose-medium were added to a mixture (50 ml) containing 5 mM phosphate buffer (pH: 6.5–7.4), 125 μM $MgCl_2$, 2–1 mM Na-citrate, 4.5 mM semicarbazide and then incubated on a shaker at 30°C for 12–5 hours. After incubation, paper chromatography of ketonic acid was carried out according to the method of Cavallini et al.[25] It was found that citrate yielded a small amount of glyoxylate by the action of the dried cells of *coli-aerogenes* bacteria under aerobic conditions as will be seen in Table X. Furthermore, the formation of glyoxylate by these bacteria was ascertained under anaerobic conditions.

Consequently, the experimental results hitherto mentioned may be regarded as a strong evidence in favour of the view that the major oxidation of tricarboxylic acids by *coli-aerogenes* becteria occurs by way of a cleavage-reaction ($C_6 \longrightarrow C_2+C_4$) and, therefore α-ketoglutarate may not be produced directly from tricarboxylic acids by the action of isocitric dehydrogenase, but may arise according to pyruvate-acetate reaction which has been propounded by the authors as follows:

$$2 \text{ Pyruvic acid} \xrightarrow{+O} \begin{array}{l}\text{Acetyl-CoA} + CO_2\\ \text{Pyruvic acid}\end{array}$$

$$\xrightarrow[\substack{\text{condensation}\\ \text{(Pyruvate-acetate}\\ \text{reaction)}}]{+O} \alpha\text{-Ketoglutaric acid} + H_2O$$

Oxidation of α-Ketoglutarate. Ajl et al. have observed that α-ketoglutarate undergoes oxidative decomposition to yield succinate by the dried cells of *E. coli*[4]. The authors carried out experiments on the oxidation of α-ketoglutarate by the bacterial cells of *coli-aerogenes*, as shown below: the washed cells of *E. coli* grown on a glucose-medium were ground with a glass at 0°C for 30 min., while in the case of *A. aerogenes* grown on a citrate-medium, the washed cells were employed.

The results obtained by the authors, in agreement with those of Ajl et al., indicate that oxidation of α-ketoglutarate actually occurs, but at a slow rate when either the ground cells (14 mg) of *E. coli* or the washed cells (5 mg) of *A. aerogenes* are incubated aerobically on a mixture (2.5 ml) containing 200 μM phosphate buffer (pH: 6.7), 4 μM $MgSO_4$, 10 μM Na-α-ketoglutarate at 37°C (Table XI). Furthermore, succinic acid was ascertained according to the method of paper-chromatography[29], to arise during the oxidation of α-

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45) J. J. R. Campbell, R. A. Smith and B. A. Eagles, *Biochim. et Biophys. Acta*, **11**, 594 (1953).

46) H. J. Satz, *Biochem. J.*, **58**, xx (1954).

47) R. A. Smith and I. C. Gunsalus, *J. Am. Chem. Soc.*, **76**, 5002 (1954).

48) J. A. Olson, *Nature*, **174**, 695 (1954).

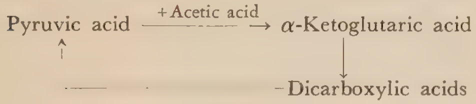
49) H. J. Satz and E. P. Hillary, *Biochem. J.*, **62**, 563 (1956).

TABLE XI

OXIDATION OF α-KETOGLUTARATE BY WASHED OR GROUND CELLS OF *COLI-AEROGENES* TYPES

Kind of bacteria	Washed cells of *A. aerogenes* grown aerobically on citrate medium	Ground cells of *E. coli* grown aerobically on glucose medium
α-Ketoglutarate added (μM)	10	10
Time of incubation (minutes)	60	155
Oxygen consumed (μM)	4.2	10.3

ketoglutaric acid when the macro-experiments were carried out with 50 ml of a reaction mixture containing 5 mM phosphate buffer (pH: 6.4), 100 μM $MgSO_4$, 1 mM Na-α-ketoglutarate and 100 mg of the dried cells. Thus, the results hitherto obtained suggest that a cyclic mechanism might occur according to which oxidation of pyruvate or acetate proceeds, as given below:



DISCUSSION

From the results of experiments with *coli-aerogenes* mentioned both in previous and present papers, the authors established the following important points.

(1) Lactic acid fermentation, which occurs anaerobically, by the action of a number of *coli-aerogenes* bacteria is converted into α-ketoglutaric acid fermentation under aerobic conditions.[21,50]

(2) The oxidative fermentation of glucose actually gives rise to a large amount of pyruvate in the following cases. Firstly, in the case of some strains of the bacteria, pyruvate is obtained in a high yield of about 10–40% of oxidized glucose.[22] The authors give them the term bacteria-inducing pyruvic acid—fermentation. Secondly, in the case of the others, pyruvate is accumulated in presence of inhibitors such as arsenic compound,[22] while the production of α-ketoglutarate occurs in their absence.[21,22] On the whole, the amounts of increasing pyruvate are observed to be nearly equivalent to those of decreasing α-ketoglutarate. Thus, these facts indicate that the formation of pyruvate may actually be an intermediate stage in α-ketoglutarate-fermentation by the bacteria.

(3) C_3-compound such as pyruvate or lactate is considered to be the most potent precursor in the production of α-ketoglutarate, the yield of this C_5-acid amounting to about 0.5–0.6 mole per two moles of C_3-acids used[23,24]

(4) A small amount of α-ketoglutarate arises during the oxidation of acetate by *E. coli*.

(5) Under aerobic conditions, a considerable amount of α-ketoglutarate was obtained with C_4-dicarboxylic acids including succinate, malate, fumarate and oxalacetate. If the C_4-dicarboxylic acid system is a component of reaction for synthesizing α-ketoglutarate, these C_4-acids must have much more potency than does C_3-acid. However, the experimental results obtained by the authors indicate that this may not be the case.

(6) The addition of acetate to the reaction mixtures which contain either C_3- or C_4-acid bring about an increase in the yield of α-ketoglutarate.

(7) *Aerobacter aerogenes* grown aerobically on a citrate-medium is capable of oxidizing citrate with an extreme readiness, but there is a very small amount of α-ketoglutarate to be detected (the maximal yield of about 0.15 mole of α-ketoglutarate per one mole of citrate), while under the same conditions the oxidative fermentation of pyruvate gives rise to a large quantity of this C_5-ketonic acid, the yield being about 0.5 mole per two moles of pyruvate.

(8) α-Ketoglutarate cannot be obtained as an intermediate product even in the case of the oxidation of *cis*-aconitate or isocitrate by *A. aerogenes* trained to citrate.

(9) It has been ascertained that citrate undergoes oxidative decomposition by the

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50) H. Katagiri, K. Imai and T. Tochikura, U.S. Patent 2786799.

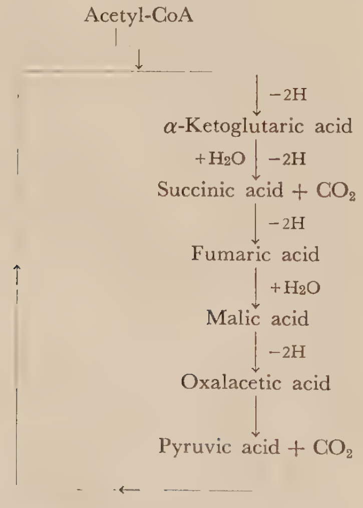
treated cells of *E. coli* grown aerobically on glucose- or glucose plus citrate-medium, but there is not any detectable amount of α-ketoglutarate as its oxidation product.

(10) It has been shown that citrate yields a small amount of glyoxylate by the dried cells of the bacteria of *coli-aerogenes* grown either on a glucose-medium or on a citrate-medium.

(11) The oxidation of α-ketoglutarate occurs at a slow rate by *coli-aerogenes* bacteria.

From the results mentioned above, the authors have concluded that the major production of α-ketoglutarate from varied carbonaceus substances (glucose, pyruvate, lactate, dicarboxylic acid) by the bacteria of *coli-aerogenes* does not proceed by way of the tricarboxylic acid cycle, that is, acetate + oxalacetate → citrate → *cis*-aconitate → isocitrate → α-ketoglutarate, but occurs by way of a certain reaction in which pyruvate and acetate are concerned. This pathway has been termed "pyruvate-acetate reaction" by the authors.[24] In this case, it seems possible that acetyl-coenzyme A is an intermediary. Moreover, investigation have led the authors to elucidate the mechanism of the oxidation of pyruvate or acetate in microorganisms.

It has been propounded by the authors that a new metabolic cycle might occur by the combination of C_4-dicarboxylic acid system and a pyruvate-acetate reaction. In this cycle, a pyruvate-acetate reaction which gives rise to α-ketoglutarate is the principal component. Therefore, the cycle is termed the α-ketoglutaric acid cycle (SCHEME I).



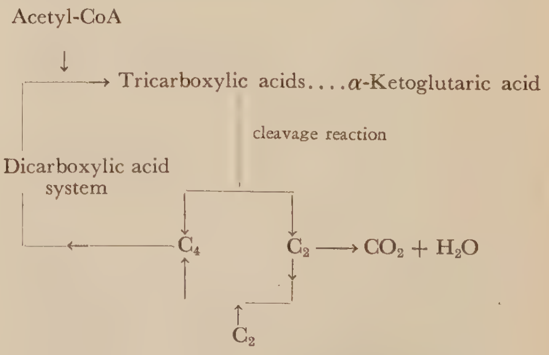
SCHEME I. α-Ketoglutaric Acid Cycle.

According to this scheme, pyruvate first reacts with acetyl-coenzyme A to form α-ketoglutarate and pyruvate is then regenerated by the oxidation of α-ketoglutarate via C_4-dicarboxylic acid system. In this case, it is evident that the net effect of one turn of this cycle is the complete oxidation of acetate.

However, it should be noted that in the case of *coli-aerogenes*, the oxidation of α-ketoglutarate does not occur with a readiness, so that α-ketoglutarate can be obtained as the major oxidation-product as was observed by the authors in the previous and the present papers.

Based on experimental results that *E. coli* incorporates labeled acetate-carbon which is externally added, into all the intermediates of the Krebs cycle within the cells, and that this organism also contains enzymes namely, aconitase and isocitric dehydrogenase, it has recently been strongly suggested by Swim and Krampitz[51], and Ajl et al.[4] that the tricarboxylic acid cycle indeed operates in *E. coli*.

From observation by many investigators that the tricarboxylic acids are splitted into



SCHEME II. Oxidation of Tricarboxylic Acids.

[51] H.E. Swim and L.O. Krampitz, *J. Bacteriol.*, **67**, 419 (1954).

C_2 and C_4 units, and also from experimental results on the bacterial production of α-ketoglutarate obtained by the authors, there are however indications that the conventional tricarboxylic acid cycle is not the sole pathway of the oxidation of pyruvate or acetate in *E. coli* (Scheme II).

Acknowledgement. This investigation has been supported in part by the Grant in Aid for Developmental Scientific Research defrayed from the Ministry of Education and by Ajinomoto Co., Inc. to which the authors' thanks are due.

[Bull. Agr. Chem. Soc. Japan, Vol. 22, No. 3, p. 153～156, 1958]

Studies on the Sunlight Flavour of Beer

Part V. The Genetical Conditions of Occurrence of Sunlight Flavour of Beer

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Received December 31, 1957

This experiment was carried out from the standpoint of genetical conditions concerning the occurrence of sunlight flavour of beer.

As a result, it is established that the essential factors causing the sunlight flavour of beer are: wort (or sucrose solution), hop materials, main fermentation attributed to yeast, and visible light.

Subsequently, this experiment was carried out to determine the component of hop which has relation to the sunlight flavour and, as a result, it was clarified that both humulone and lupulone are related to the sunlight flavour.

The fact that an unpleasant flavour occurs on exposing beer to sunlight was first referred by C. Lintner in 1875.1) Thereon, many studies on sunlight flavour of beer have been performed: i.e., J. de Clerck2) clarified that redox-potential drops when beer is exposed to sunlight, and a few years afterwards, F. Mendlik3) reported that the flavouring material occurring on sunlight is a volatile substance. More recently, quantitative determination of hydrogen sulfide and volatile mercaptan of beer was performed by M. W. Brenner4) et al., their conclusion being that the amount of volatile mercaptan slightly increases when beer is exposed to sunlight.

However, despite the above-mentioned investigations, no assertion has yet been made in concern of the occurrence of sunlight flavour of beer. In respect of this, several model experiments have been carried out by one of the present authors,5) this report given

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1) C. Lintner, Lehrbuch der Bierbrauerei (1875).

2) J. de Clerck, Wochenschrift f. Brauerei 378 (1934).

3) F. Mendlik, Wochenschrift f. Brauerei 393 (1938).

4) M.W. Brenner et al., Am. Brewer., **87**, No. 3, 29 (1954); **88**, No. 1, 43; No. 2, 48 (1955).

5) Y. Obata and T. Yamanishi, *Agriculture (Japan)*, **9**, 537 (1947); *Bull. Chem. Soc. Japan*, **22**, 247 (1949); *J. Agr. Chem. Soc. Japan*, **23**, 154 (1950); *Bull. Chem. Soc. Japan*, **23**, 125 (1950); *J. Agr. Chem. Soc. Japan*, **24**, 477 (1951);
Y. Obata and H. Horitsu, *J. Agr. Chem. Soc. Japan*, (in press).

here is one of this series. The experiment has been performed from the standpoint of genetical conditions concerning the occurrence of sunlight flavour of beer, in order to confirm these respects.

The present authors concluded that there are four essential factors related to the occurrence of sunlight flavour:

1. Wort or sucrose solution, the former being able to be substituted by the latter and other fermentable carbohydrates
2. Hop materials
3. Main fermentation attributed to yeast
4. Visible light at the wave length during 400–450 mμ.

If even one of the above-mentioned four factors is lacking, sunlight flavour does not occur. Moreover, in order to ascertain the component of hop which is related to the occurrence of sunlight flavour, hop was fractioned; and, it was confirmed that humulone and lupulone are both related to the occurrence of sunlight flavour of beer.

EXPERIMENTAL

Conditions for occurrence of sunlight. This experiment was first conducted in order to specify the conditions, under which sunlight flavour occurs, involving the following four factors: wort and hop as a raw material of beer, fermentation attributed to beer yeast, and sunlight exposure. Scheme 1. indicates the relation of the above-mentioned four factors.

From Scheme 1., it was recognized that four essential factors are related to the occurrence of sunlight flavour. Wort or 10% sucrose solution (the latter be described below), hop materials, fermentation attributed to yeast, and visible light. Subsequently, each factor was investigated more detailly.

Wort	Hop	Fermentation	Exposure	Sunlight flavour
+	−	−	+	×
+	−	+	+	×
−	+	−	+	×
+	+	−	+	×
+	+	+	−	×
+	+	+	+	○

Notes: −, no addition
+, addition
×, non-occurrence of sunlight flavour
○, occurrence of sunlight flavour

SCHEME 1. Essential Factors for Occurrence of Sunlight Flavour.

(1) Wort, which is the substrate, was examined. Wort prepared by the action of malt ferment, consists of a number of components containing carbohydrate and other substances. However, it was necessary for the studies on the genetical condition of sunlight flavour to evade complexity owing to several materials besides carbohydrate. Therefore, wort was substituted by 10% sucrose solution solely consisting of carbohydrate substance, the other conditions left unchanged. According to this way, beer was prepared and exposed to sunlight for 2 hours, and an organoleptic test was carried out. It was made clear that sunlight flavour also occurs under these conditions, indicating that the active material of wort is carbohydrate.

(2) The experiment was performed on sunlight. Details concerning the experiment, are reported in the third report by one of the present authors. Beer was injected into four kinds of colouring bottles, and exposed to sunlight for 2 hours. The organoleptic test was then carried out; the bottles were broken and absorption spectra on the pieces of each bottle was estimated, the pieces being 6 mm-thick.

The result obtained is shown in Fig. 1.

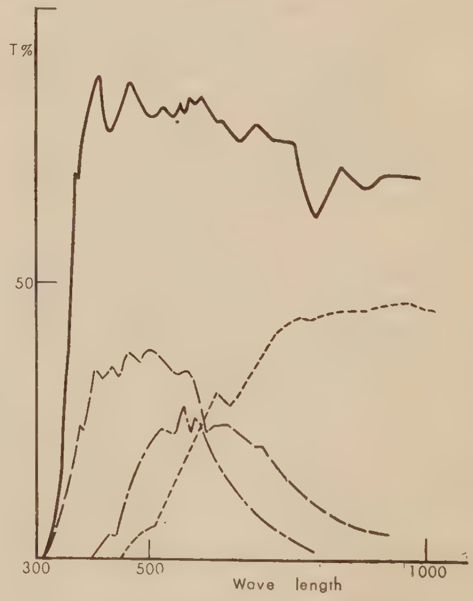

FIG. 1. Absorption Spectra of Beer Bottles.

——, colourless —·—, green
---, blue ·····, brown

Sunlight flavour occurs, as shown in Fig. 1., in case of colourless, blue, and green bottles, but does not in case of a brown bottle.

It is therefore clarified that the influential wave length lies between 400–450 $m\mu$. In concern of the influence of ultraviolet ray, as common glasses absorb the corresponding wave length, quartz glass irradiated by a mercury lamp (200 W) 50 cm in distance for 2 days, was used in place of the former, but occurrence of sunlight flavour was not observed; thus, it is clear that the active ray is in the range of visible wave length. As for the fluorescent lamp, the majority of its energy consists in the extent of 400–600 $m\mu$; consequently, 2–4 days were required by the fluorescent lamp (100 W) 50 cm in distance, to obtain the same result in case of sunlight.

(3) In the case of addition to wort only hop without yeast, the sunlight flavour does not occur even when it is exposed to sunlight for 2 hours, so it is clear that fermentation is necessary for sunlight flavour. Moreover, as for its process, the sunlight flavour occurs even in case of only the main-fermentation at 0–5° for 10 days.

(4) The following experiment was carried out with hop which is characteristic of beer. Naturally, as the hop component is not simple, the experiment was conducted on the extractions by various solvents: first, the hop was extracted with ether, subsequently with ethanol, and an unextracted residue remained. Then, three kinds of beer containing only each of the above-mentioned extracts and the residue at a concentration of 2 g/*l* were prepared, and exposed to sunlight for 2 hours, and organoleptic test was carried out at each time.

It was found that it is only the ethereal material of hop which is related to the flavour. Subsequently, in order to ascertain whether it is volatile or the non-volatile fraction that causes sunlight flavour, steam distillation was carried out; resulting that, the non-volatile fraction was found to have relation. This means that the occurrence of flavour is not caused by the decompositive product of essential oil or other volatile materials.

Fractionation of hop. Fractionation was carried out on the ether extract of hop. The ethereal extract was first extracted with 5% $NaHCO_3$ to separate strong acid-fraction, then with $2N\text{-}Na_2CO_3$ to separate the weak acid-fraction, 2N-NaOH to separate phenol-fraction, 2N-HCl to separate base-fraction, and finally, a neutral fraction remained.

Next, five kinds of beer containing only each of the above-mentioned extracts, at a concentration of 500 mg/*l* were prepared, and exposed to sunlight for 2 hours, and organoleptic test was carried out.

It was found that only the phenol-fraction has relation to the flavour. This means that characteristic components of hop, that is, humulone and lupulone have relation to the flavour.

Separation of α-, β-soft, and γ-, resins. The following experiment was carried out in connection with this. There are three kinds of hop resins: α-, β-soft, and γ-, resins. Lead-precipitable fraction of resin is commonly known as the α-soft resin of hop. α-soft resin mainly consists of humulone, which can become crystalline, and a non-lead precipitable fraction of resin is known as the β-soft resin of hop. β-soft resin mainly consists of lupulone, which can become crystalline.

In addition to the above-mentioned two crystalline resins, there is another non-crystalline resin, commonly known as γ-resin. The isolation of resin was thus carried out. At first, the above-mentioned phenol-fraction was extracted with *n*-hexane, and then divided into two fractions: the soluble-fraction which consisted of $(\alpha+\beta)$-soft resins (humulone and lupulone) and the insoluble-fraction which consisted of γ-resin. Then, the beer containing only each of the above-mentioned fraction at a concentration of 250 mg/*l* was prepared, and exposed to sunlight for 2 hours, and organoleptic test was performed. This revealed that the $(\alpha+\beta)$-soft resins of hop are related to sunlight flavour while γ-resin is not.

Separation of α-, and β-, soft resins. In order to ascertain whether it is α-, or β-soft resin which causes the flavour, separation of these resins was carried out under the following process: $(\alpha+\beta)$-soft resins were dissolved into MeOH, and MeOH solution containing Pb-Acetate was added to the MeOH solution of $(\alpha+\beta)$-soft resins, which produced the precipitate of α-soft resin-Pb-complex. Next, the complex-precipitate was decomposed with H_2SO_4, and extracted with ether.

The process was repeated twice, then a *o*-phenylenediamine-complex of humulone was formed by the addition of benzene solution of *o*-phenylenediamine. This process was also twice repeated. After purification, the complex was decomposed with HCl, then free humulone was obtained. The crystalline humulone thus obtained, gives *o*-phenylenediamine-complex of m.p. 117°, its absorption spectrum shown in Fig. 2. Subsequently, MeOH solution evading the humulone

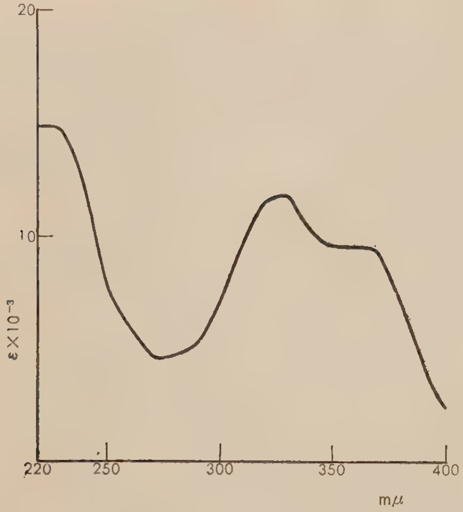

FIG. 2. Absorption spectrum of humulone (alkali-MeOH sol'n).

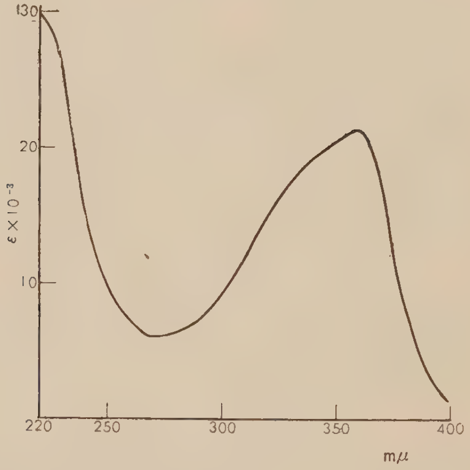

FIG. 3. Absorption spectrum of lupulone (alkali-MeOH sol'n).

from the mixture, was decomposed with H_2SO_4 and concentrated in vacuo, and then, the crystalline lupulone was isolated.

The crystalline lupulone thus obtained gave m.p. 92°, and the absorption spectrum shown in Fig. 3. It must be added that these results concerning the above-mentioned two components, that is, humulone and lupulone are identical with those described by Harris[6].

Finally, the beer containing only each of the above-mentioned humulone and lupulone at a concentration of 250 mg/*l*, was prepared, and exposed to sunlight for 2 hours, and organoleptic test was performed at each time. As a result, it was established that both humulone and lupulone are related to the occurrence of sunlight flavour of beer.

The authors wish to acknowledge their indebtedness to Nippon Beer Company and Kirin Beer Company for supplying the samples.

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6) G. Harris, *J. Chem. Soc.*, **1952**, 1906.

[Bull. Agr. Chem. Soc. Japan, Vol. 22, No. 3, p. 157～162, 1958]

On the Susceptibility of the Crystalline and Retrograded Forms of Amylose of Different Molecular Weights toward Black-koji Amylase System

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Received January 6, 1958

The crystalline and retrograded amylose preparations were digested by black-koji amylase system in the heterogeneous state such as in the case of raw starch digestion. It was found that the usual retrograded amylose should be fractionated into two parts—one easily digestible and the other resistant toward black-koji amylase system. The latter part, when recrystallized by *n*-butanol, however, turned highly susceptible toward the enzyme action. Its molecular weight, as determined by the light scattering method, was found to be 32,000, a value much smaller than that of the original amylose (140,000).

INTRODUCTION

There are many reports dealing with the retrograded starch and its resistance to enzyme action. As early as in 1903, Maquenne[1] reported retrograded starch is not attacked by malt diastase hence, the extent of the retrogradation was determined by measuring the decrease in the amount of starch saccharified by malt amylase. Using a similar analytical technique, except substituting ptyalin for malt amylase, Sallinger[2] found that a solution of Lintner soluble starch retrograded rapidly at 7°C. Whistler and Johnson[3] demonstrated that as the average molecular size of potato amylose was reduced by mild acid hydrolysis, the extent of retrogradation during a 24 hour-period increased to its maximum and later decreased. From this fact, they suggested that for amlyose molecules there exists a critical molecular size for which retrogradation rates are at a maximum. Molecules of a larger size than the critical retrograde more slowly, presumably being due to the steric effects resulting from their more coiled or kinked structure. On the contrary, molecules of smaller size retrograde more slowly, presumably being due to the increased solubility inherent in a lower molecular weight molecule. Recently, Lansky et al.[4] claimed that the tapioca A-fraction does not retrograde because its chain molecule is too long to permit ready orientation to an aggregated state. The molecular weight of those amyloses, however, was only surveyed by intrinsic viscosity and reducing value qualitatively.

On the other hand, one of the authors[5] has reported on the digestion of various kinds of raw starch by black-koji amylase system, and found that the saccharogenic amylase fraction hydrolyzed raw glutinous starches easily, but only weakly in case of non-glutinous starches. This fact seems to suggest that there will exist some relationships between the existent conditions of amylose in the starch granule and its digestibility.

In the present paper, amyloses prepared in the crystalline and retrograded forms were subjected to digestion in the heterogeneous state, by black-koji amylase. Furthermore, the digestibility of amylose in both forms of different molecular weight were compared. The molecular weights of amyloses were mined by the light scattering method.

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[1] L. Maquenne, *Compt. rend.*, **137**, 88, 797, 1266 (1903).

[2] H. Sallinger, *Kolloid Z.*, **25**, 111 (1919).

[3] R. L. Whistler and C. Johnson, *Cereal Chem.*, **25**, 418 (1948).

[4] S. Lansky, M. Kooi and T. J. Schoch, *J. Am. Chem. Soc.*, **71**, 4066 (1949).

[5] S. Ueda, *J. Agr. Chem. Soc. Japan*, contribution.

EXPERIMENTAL

Preparation of Black-koji Amylase Solution: As reported previously[5)], black-koji amylase solution was prepared from the shaking culture of *Aspergillus awamori* (Kawachi strain).

Preparations of Amylose.

(1) Crystalline amylose: Crystalline amylose was prepared from potato starch by the "precipitant-enzyme method" devised by Nikuni et al.[6)] After the addition of 2.2 ml of *n*-caprylic acid to about 2 *l* of 2 per cent hot potato starch paste, it was cooled down to room temperature. One-tenth N acetate buffer, pH 4.8, (100 ml) and 0.6 per cent Taka-diastase 100 ml were added to the starch paste and the paste digested for a period of 3 hours at room temperature. The enzymatic action was stopped by rapid heating and the digested solution, after it was added with active carbon, was filtered with suction in a hot state. On cooling the clear filtrate after further addition of 0.8 ml *n*-caprylic acid, disk-shaped crystals of amylose-caprylic acid complex separated out. After the crystals were collected by centrifugation and washed twice with *n*-butanol saturated water, they were dissloved in 2 *l* of distilled water and 200 ml of *n*-butanol was added to the solution. After heating to boil, the solution was gradually cooled down to room temperature. The recrystallized complex (dia 6 μ petal-shaped) was collected, washed, dried and weighed (Amylose-I). The yield of amylose was 7–8 per cent of the potato starch.

(2) Retrograded amylose: Five g of Amylose-I was dissolved in 100 ml 1N NaOH in the icebox. After filtration through glass filter No. 4, the filtrate was neutralized with 1N HCl to about pH 5.0 and allowed to retrograde for three days in the icebox. Then, the amylose retrograded and precipitated to the bottom. Collecting by centrifugation, the retrograded amylose was washed with cold distilled water until the iodine coloration of the washings disappeared (Retrograded Amylose-I).

This retrograded amylose was digested in the heterogeneous state by black-koji amylase solution until the digestion proceeded no more, and the remainder was washed and dried (Retrograded Amylose-II).

This Retrograded Amylose-II was treated once more by dissolving in alkali and retrograding in the icebox (Retrograded Amylose-III).

(3) Crystallization of retrograded amylose: Retrograded amylose could not be crystallized by the method usually employed because of its instantaneous precipitability in the non-alkaline solution. The following device gave way to successful crystallization of retrograded amylose. After dissolving 1 g of dry powder of the Retrograded Amylose-II in 250 ml 1N NaOH, the solution was filtered through glass filter No. 4 and neutralized with 1N HCl which was previously added with one-tenth in volume of *n*-butanol in order to avoid the momentary precipitation of amylose. After adding one-tenth in volume of *n*-butanol, the whole solution was first heated to boil and gradually cooled down to room temperature for a period of six hours. Thus, rectangular crystals of amylose-butanol complex ($11 \times 7 \mu$) were obtained.

Digestion of Retrograded and Crystalline Amyloses by Black-koji Amylase System.

(1) Action on Retrograded Amylose.

(a) Heterogeneity of Retrograded Amylose:

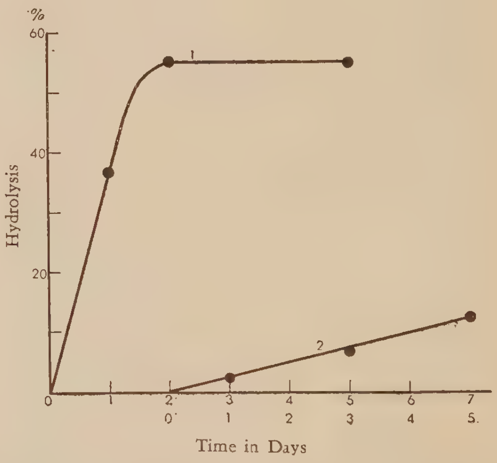


FIG. 1. Hydrolysis of Retrograded Amylose by Black-koji Amylase System.

Curve 1; Hydrolysis-time curve of the retrograded amylose prepared directly from Amylose-I.

Curve 2; Hydrolysis-time curve of the re-retrograded amylose prepared from resistant half to black-koji amylase of retrograded amylose.

[6)] Z. Nikuni, K. Takaoka and H. Fuwa, *J. Agr. Chem. Soc. Japan*, **26**, 216 (1952).

Amylose-I (0.1g) was dissolved in 5 ml 1N NaOH and neutralized with 1N HCl and placed in the icebox for three days. The amylose retrograded and precipitated on the bottom of the flask. To this flask which contained retrograded amylose in the precipitated form, were added black-koji amylase solution 5 ml, M/5 citrate buffer (pH 3.6) 5 ml and toluene 1 ml and the reaction mixture incubated at 30°C for digestion. As shown in Fig. 1, the hydrolysis-time curve showed a distinct break at about 50 per cent hydrolysis (curve 1). In order to investigate whether the remained retrograded amylose was inherently resistant or became resistant during the digestion with black-koji amylase, the following experiment was carried out. After Amylose-I was retrograded and digested by black-koji amylase for two days as described above, the remained retrograded amylose was collected, washed, dissolved in 5 ml 1N NaOH, neutralized with 5 ml 1N HCl and retrograded once more in the icebox for three days. To this retrograded amylose suspension, were added 5 ml of black-koji amylase solution, 5 ml of M/5 citrate buffer (pH 3.6) and 1 ml of toluene and the reaction mixture similarly incubated at 30°C as before.

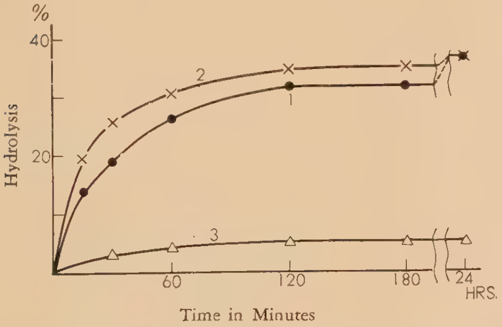


FIG. 2. Hydrolysis of Retrograded Amylose by Black-koji Amylase System.

Curve 1 ; Retrograded Amylose-I.
Curve 2 ; Retrograded Amylose-I. (The concentration of the amylase was used twice in the Curve 1.)
Curve 3 ; Retrograded Amylose-III.

As shown in curve 2 in Fig. 1, the re-retrograded amylose was found to be extremely resistant against black-koji amylase. From these results it is plausible to consider that the retrograded amylose should be fractionated into two fractions, one which is easily digested by black-koji amylase, the other very resistant to the amylase.

(b) Action of Black-koji Amylase on Retrograded Amylose-I and -III: Retrograded Amylose-I was directly prepared from Amylose-I. Retrograded Amylose-III was prepared by re-retrogradation of the resistant half after digesting Retrograded Amylose-I with black-koji amylase.

The compositions of the reaction mixtures were as follows:

Reaction mixture I	
Retrograded Amylose-I	10 ml
(amylose 134.0 mg as glucose)	
Black-koji amylase solution	5 ml
N/10 acetate buffer (pH 3.6)	10 ml
Distilled water	5 ml
Reaction mixture II	
Retrograded Amylose-I	10 ml
(amylose 134.0 mg as glucose)	
Black-koji amylase solution	10 ml
N/10 acetate buffer (pH 3.6)	10 ml
Reaction mixture III	
Retrograded amylose-III	5 ml
(amylose 31.0 mg as glucose)	
Black-koji amylase solution	5 ml
N/10 acetate buffer (pH 3.6)	5 ml

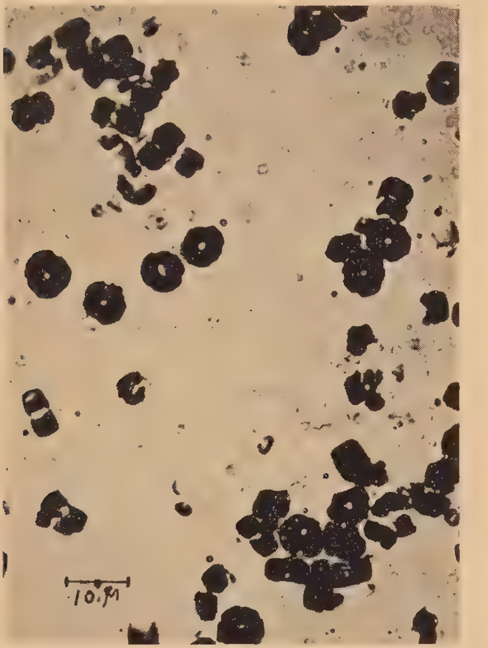
These reaction mixtures were incubated at 30°C for digestion. As shown in Fig. 2, the rate and extent of hydrolysis of Retrograded Amylose-I showed a distinct break at 30 per cent hydrolysis and afterwards the digestion proceeded very slowly up to 35 per cent (curves 1 and 2). Much difficulty was found in hydrolysis of Retrograded Amylose-III and the extent of hydrolysis amounted up to only 5 per cent. These facts again confirmed that the retrograded amylose could be separated into two fractions, i. e., the resistant and the non-resistant to black-koji amylase. Difference in the extent of hydrolysis in Figs. 1 and 2 (50% and 35%) seems to be attributed to the loss of digestible retrograded amylose by the washing of Retrograded Amylose-I in the case of Fig. 2.

(2) Action of Black-koji amylase on the Crystalline Amylose.

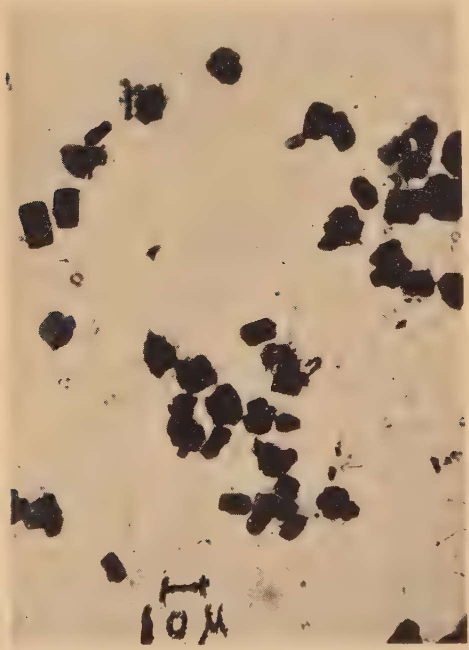
Crystalline Preparations from Amylose-I and those from Retrograded Amylose-II represented different shapes of crystals as shown in photographs 1 and 2.

The compositions of the reaction mixtures were as follows:

Reaction mixture I	
Crystalline Amylose-I suspension	5 ml
N/10 acetate buffer (pH 3.6)	5 ml
Distilled water (*n*-butanol saturated)	4 ml
Black-koji amylase solution	5 ml
n-butanol	1 ml



PHOTOGRAPH 1. Crystalline Amylose-Butanol Complex from Amylose-I.



PHOTOGRAPH 2. Crystalline Amylose-Butanol Complex from Retrograded Amylose-II.

Reaction mixture II	
Amylose solution*	10 ml
N/10 acetate buffer (pH 3.6)	5 ml
Black-koji amylase solution	5 ml
Reaction mixture III	
Crystalline amylose from Retrograded Amylose-II	10 ml
N/10 acetate buffer (pH 3.6)	10 ml
Black-koji amylase solution	8 ml
n-butanol	2 ml

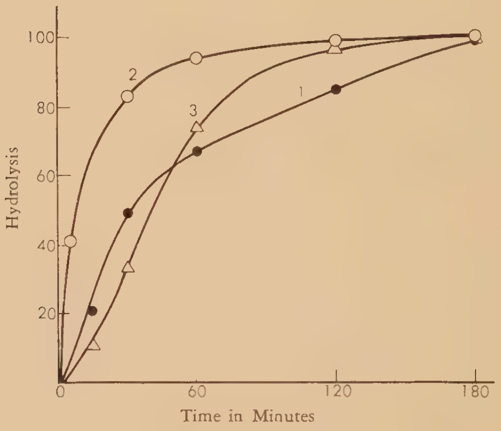


FIG. 3. Hydrolysis of Crystalline Amylose by Black-koji Amylase System.

Curve 1; Hydrolysis of the suspension of crystalline amylose prepared from Amylose-I.

Curve 2; Hydrolysis of the solution of crystalline amylose prepared from Amylose-I.

Curve 3; Hydrolysis of the suspension of crystalline amylose prepared from Retrograded Amylose-III.

As shown in Fig. 3, the crystalline amylose (curves 1 and 3) was converted to glucose as smoothly as amylose solution (curve 2), though the rates of hydrolysis slightly differed from each other.

It is very interesting to note that retrograded amylose, which could not be attacked by the black-koji amylase in the retrograded state, changed to be highly susceptible to the enzymic action in the crystalline state.

Determination of Molecular Weights by the Light Scattering Method.

Molecular weights of Amylose-I and of Retrograded Amylose-II were determined by this method.

Amylose preparation was dissolved in N/3 NaOH. After centrifugation for 2 hours at 12,000 r.p.m.

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* Five ml of distilled water was added into 5 ml crystalline amylose suspension to dissolve the crystals.

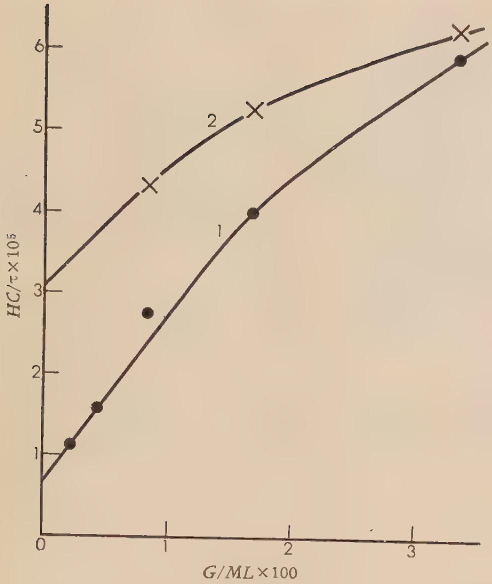

FIG. 4. Determination of $\underset{C\to0}{HC/\tau}$ by the Light Scattering Method.

Curve 1; $HC/\tau-C$ curve of Amylose-I.
Curve 2; $HC/\tau-C$ curve of Retrograded Amylose-II.

(14,500 ×g) in order to remove the dust, the supernatant was used for measuring the refractive index* and the intensity of light scattering**.

Molecular weight was calculated for each preparation, using the following equation derived by Debye[7].

$$M=\frac{\tau}{HC}$$

$$H=\frac{32\pi^3 n_0^2}{3N\lambda^4}\left(\frac{n-n_0}{C}\right)^2$$

τ; excess turbidity, M; molecular weight, C; concentration in grams per ml, H; a proportionality constant, n; refractive index of the solution, n_0; refractive index of the solvent, N; the Avogadro number, λ; wave length of the light used.

In the case of Amylose-I,

C	0.0333	0.0167	0.0083	0.0042	0.0021
$HC/\tau\times10^5$	5.91	3.99	2.75	1.592	1.126

Therefore, $C\to0$ $HC/\tau=0.67\times10^{-5}$ M.W.$=140,000$

In the case of Retrograded Amylose-II,

C	0.0333	0.0167	0.0083	0.0042	0.0021
$HC/\tau\times10^5$	6.25	5.28	4.35	(7.63)***	(29.3)***

Therefore, $C\to0$ $HC/\tau=3.1\times10^{-5}$ M.W.$=32,000$

DISCUSSION

Heretofore, it has been shown by many workers that the average size of the amylose molecules plays an important role in the determination of the rate at which an amylose retrograded, but the actual molecular weight of amylose liable to retrograde has yet not been determined accurately.

From the experiments above mentioned, it became clear that retrograded amylose could be fractionated into two parts, one of which was extremely resistant to the amylase in the retrograded state, while the other was not.

The former fraction, resistant to the amylase, after being dissolved in alkaline solution and precipitated once more by neutralization, was found to be extremely resistant to the amylase as in the previous case. However, when it was crystallized as butanol complex, this amylose very liable to retrograde was found to be readily digested by the amylase. Molecular weight of this amylose (32,000) was smaller than that of the original amylose (140,000) when determined by using the light scattering method. This fact so far coincides well with suggestions of other workers.

Though many discussions[8,9,10,11] appear about the glucosidic linkages of amylose molecules from the enzymatic degradation point of view, it should be considered that there is a certain molecular state of amylose, as described above, in which amylose retrogrades instantaneously and too rigidly to attack enzymatically.

As previously reported[5], the authors observed the different digestibility of various native starch granules caused by black-koji amylase, which widely varied between two extremities, that is, potato starch which was most resistant to digestion and glutinous cereal starches which showed the lest resistance.

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[7] P. Debye, *J. Appl. Phys.*, **17**, 392 (1946).

* Phoenix's Differential Refractometer.

** Brice Phoenix's Instrument employed for measuring light scattering.

*** These values should be excluded from calculation because of the considerable influence of the turbidity.

[8] S. Peat, S. J. Pirt and W. J. Whelan, *J. Chem. Soc.*, **1952**, 705.

[9] R. H. Hopkins and R. Bird, *Nature*, **172** 492 (1953).

[10] A. Baba and H. Kojima, This Bulletin, **19**, 167 (1955).

[11] E. F. Neufeld and W. Z. Hassid, *Arch. Biochem. Biophys.*, **59**, 405 (1955).

Considering these facts on the light of variability of amylose in the digestibility by the amylase as described above, it seems highly plausible to assume that the amount and, especially the nature of existanceof amylose would be one of the determining factors of resistance of raw starch to enzymic amylolysis.

CONCLUSION

(1) Amylose, in its retrograded form, was hydrolyzed by the black-koji amylase to glucose up to the extent of about 50 per cent with the exception of the indigestible part, the molecular weight of which was determined as 32,000, while that of the original amylose as 140,000.

(2) Notwithstanding the size of molecular weight, the amylose when crystallized as butanol complex was found to be hydrolyzed easily by black-koji amylase.

Acknowledgement The authors wish to thank Mr. K. Hayashi and Miss J. Oda for their invaluable advices through the determination of molecular weight of amylose by means of the light scattering method.

This work was aided in part by a grant from the Scientific Research Fund, Ministry of Education. (Composite research subject: Fundamental studies on the utilisation of starch. Representative: Zirô Nikuni) The authors' thanks are also due to Professor Z. Nikuni.

[Bull. Agr. Chem. Soc. Japan, Vol. 22, No. 3, p. 162~168, 1958]

Geometrical Isomers of α-Methylmuconic Acid

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Received January 6, 1958

Peracetic acid oxidation of *o*-cresol gives α-methyl-*cis, cis*-muconic acid (II), m.p. 189–90°. This is lactonized with sulphuric acid to the lactonic acid (IV), which is converted to the lactonic methyl ester (V). The treatment of the lactonic ester with sodium methoxide affords (δ)-methyl (α)-hydrogen α-methyl-*cis, trans*-muconate (XI), which in turn is hydrolyzed to α-methyl-*cis, trans*-muconic acid (XII), m.p. 172°. The *cis, cis*- and *cis, trans*-acids are isomerized to the *trans, trans*-isomer (XIV), m.p. 273°, by boiling with 20% aqueous sodium hydroxide. The geometrical configurations of these isomers are established by synthetic evidence and spectral data.

The authors have recently reported[1] on the geometrical configuration of $\alpha\delta$-dimethylsorbic acid. Now, the authors have investigated α-methylmuconic acids as one of the polyene compounds of stereochemical interest. Kuhn and Michel[2] prepared an isomer of α-methylmuconic acid by the condensation of ethyl tiglate with ethyl oxalate, followed by acetylation and hydrolysis. However, the geometrical configuration of the acid has not yet been confirmed, though it is assumed tentatively to be *trans-trans*.

α-Methylmuconic acid can exist theoretically in four geometrically isomeric forms *cis-cis*,

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[1] Y. Inouye, T. Sugita and M. Ohno, a) *This Bulletin*, **21**, 5 (1957), *Botyu-Kagaku*, **21**, 86 (1956), b) *This Bulletin*, **21**, 224 (1957), *Botyu-Kagaku*, **22**, 227 (1957).

[2] R. Kuhn and J. Michel, *Ber.*, **71**, 1119 (1938)

cis-trans, *trans-cis* and *trans-trans*.* Of these, three isomers were obtained by the present authors through a novel route of synthesis and their geometrical configurations were established. The isolation of the remaining one isomer, *trans-cis*, has failed in spite of any attempt.

The oxidation of *o*-cresol (I) with peracetic acid gave α-methyl-*cis*, *cis*-muconic acid (II) m.p. 189–90°, which was converted with diazomethane into the dimethylester (III), m.p. 35°.

The treatment of the *cis*, *cis*-acid (II) with cold 80% sulphuric acid gave the unsaturated lactonic acid (IV), m.p. 105–6°, the structure of which is evidenced by the subsequent chemical processes. The lactonization was also effected by boiling with water, yielding the same compound (IV). It was of interest that the lactonization of the acid (II) under any reaction conditions employed, gave exclusively the lactonic acid (IV) alone, in contrast to the authors' expectation; this seems likely, from the theoretical considerations which will be discussed later.

The lactonic acid (IV) afforded a liquid methyl ester (V) with diazomethane. The unsaturated lactonic ester (V) took up one mole. hydrogen on catalytic hydrogenation, giving the saturated lactonic ester (VI), m.p. 45–6°. The treatment of the saturated lactonic ester (VI) with sodium methoxide in methanol gave an open-chain isomer (VII), hydrolysis of which afforded α-methyl-Δ^{γ}-dihydromuconic acid (VIII), m.p. 121–2°. The half ester (VII) was characterized by ozonolysis to yield methyl hydrogen oxalate (IX) (as itself, and was easily hydrolyzed to oxalic acid) and methylsuccinic acid (X) (as itself and as *p*-phenylphenacyl ester, m.p. 179°). Therefore, the structures of the unsaturated lactonic acid and of the compounds derived therefrom, were confirmed as IV–VIII.

The treatment of the unsaturated lactonic ester (V) with sodium methoxide in methanol gave the expected (δ)-methyl (α)-hydrogen α-methyl-*cis*, *trans*-muconate (XI), m.p. 121°, which was readily reconverted into the parent unsaturated lactonic ester (V) by distillation under reduced pressure. On hydrolysis with dilute sodium hydroxide, the half-ester (XI) produced α-methyl-*cis*, *trans*-muconic acid (XII), m.p. 172°. Esterification of either the acid (XII) or the half-ester (XI) with diazomethane yielded the same dimethyl ester (XIII), m.p. 60°.

When the *cis*, *cis*- (II) and the *cis*, *trans*-acids (XII) were heated with 20% sodium hydroxide, these were equally isomerized into the *trans*, *trans*-form (XIV), m.p. 273°, which might be identical with that obtained by Kuhn and Michel[2], m.p. 276°, though direct comparison by mixed melting point is lacking. The acid (XIV) was converted with diazomethane into the dimethyl ester (XV), m.p. 55.5°, which also might correspond to the ester recorded by Harris and Binns[3], m.p. 46–7°.

On catalytic hydrogenation over platinum catalyst, each of the *trans*, *trans*-, *cis*, *trans*- and *cis*, *cis*-acids took up two moles. hydrogen and gave the same α-methyladipic acid (XVI), m.p. 58–9°, which was also characterized by its diamide, m.p. 185–6°.

Configuration: The configurations of the three acids (II), (XII) and (XIV) followed unambiguously from the method of preparation and the spectral data. The high melting and the most stable isomer (XIV) is expected to have all *trans*-configuration, because it is also obtained by the condensation of tiglate of the well-defined *trans*-configuration with oxalate, in which the *trans*-configuration is favoured in general. The failure of the isomer to be lactonized with 80% sulphuric acid into the lactonic acid, is in good agreement with this deduction.

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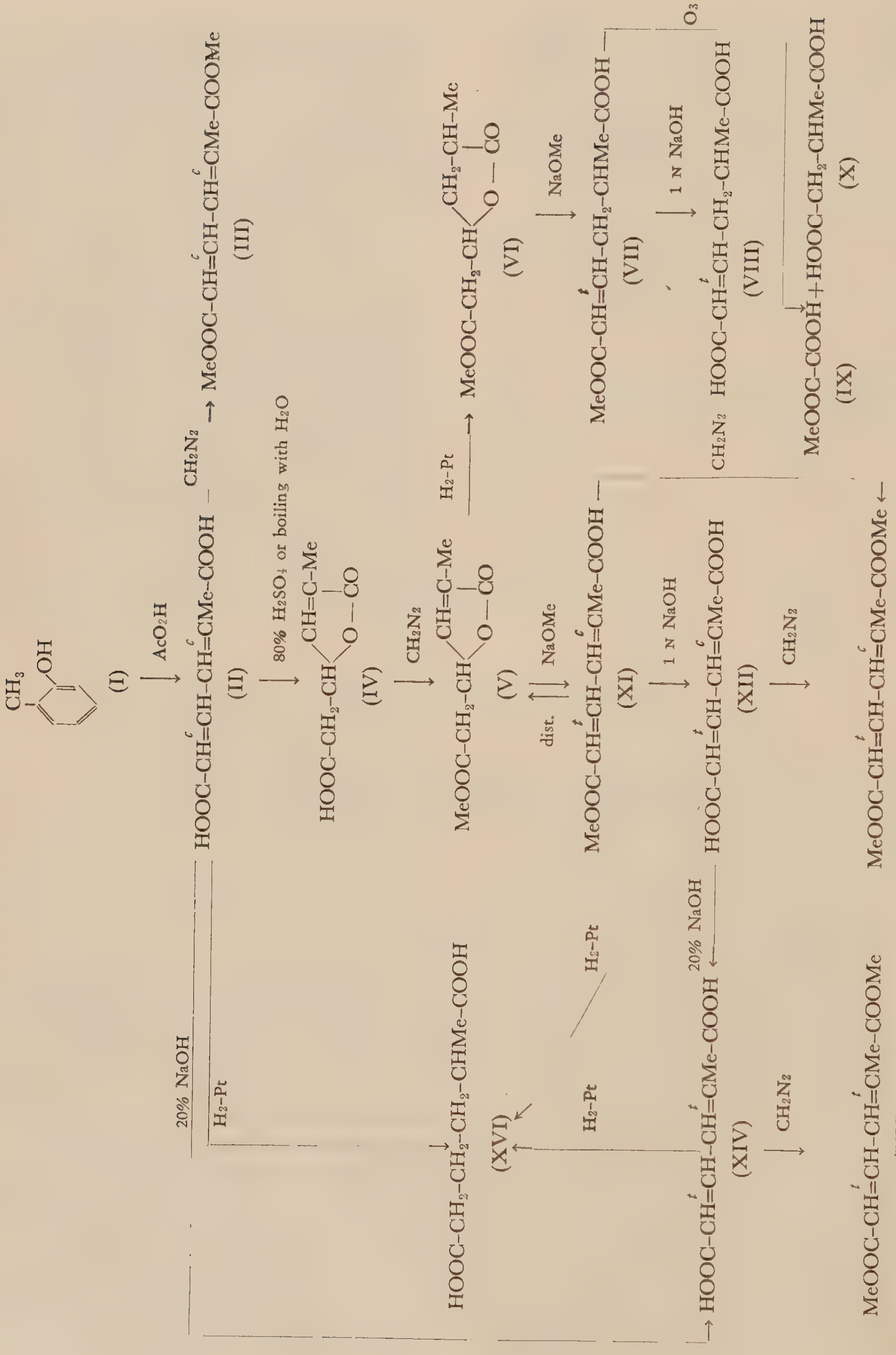
* The nomenclature of these compounds are based on α-methylmuconic acid, therefore, e.g., $MeOOC-\overset{trans}{CH=CH}-\overset{cis}{CH=CMe}-COOH$ is named (δ)-methyl (α)-hydrogen α-methyl-*cis*, *trans*-muconate. The terms *cis* and *trans* are given in positional order, thence the first term indicates the configuration of the αβ-double bond.

[3] J. O. Harris and F. Binns, *Nature*, **179**, 475 (1957).


CH3
OH
(I)
AcO2H
HOOC-CH=CH-CH=CMe-COOH
c
c
(II)
CH2N2
MeOOC-CH=CH-CH=CMe-COOMe
(III)
80% H2SO4 or boiling with H2O
HOOC-CH2-CH
CH=C-Me
O — CO
(IV)
CH2N2
MeOOC-CH2-CH
CH=C-Me
O — CO
(V)
H2-Pt
MeOOC-CH2-CH
CH2-CH-Me
O — CO
(VI)
NaOMe
MeOOC-CH=CH-CH2-CHMe-COOH
t
(VII)
1 N NaOH
HOOC-CH=CH-CH2-CHMe-COOH
(VIII)
O3
MeOOC-COOH + HOOC-CH2-CHMe-COOH
(IX)
(X)
CH2N2
dist.
NaOMe
MeOOC-CH=CH-CH=CMe-COOH
(XI)
1 N NaOH
HOOC-CH=CH-CH=CMe-COOH
(XII)
CH2N2
MeOOC-CH=CH-CH=CMe-COOMe
(XIII)
20% NaOH
H2-Pt
HOOC-CH2-CH2-CH2-CHMe-COOH
(XVI)
H2-Pt
H2-Pt
20% NaOH
HOOC-CH=CH-CH=CMe-COOH
(XIV)
CH2N2
MeOOC-CH=CH-CH=CMe-COOMe
(XV)

The acid (II) obtained by the oxidative ring-fission of *o*-cresol is most likely to have *cis, cis*-configuration. It is, in fact, readily lactonized to IV. Moreover, it is isomerized to the high melting acid (XIV) both directly and *via* the third acid (XII).

The half-ester of the third acid (XI) produced by ring opening reaction with alkoxide is expected to have *cis, trans*-configuration. The $\alpha\beta$-double bond must be undoubtedly *cis* in the lactone. The *cis*-configuration of the $\alpha\beta$-double bond should be retained as such during the ring opening reaction, since the resulting half-ester was capable of ready lactonization into the parent compound (V). The $\gamma\delta$-double bond which is created by the ring opening reaction, is considered to have *trans*-configuration by the analogies in similar reactions.

The third acid (XII) can also be isomerized to the *trans, trans*-acid (XIV). It is evident, therefore, that the third acid (XII) has *cis, trans*-configuration.

The first acid (II) produced by ring fission of *o*-cresol has at least one *cis*-double bond, since it is readily lactonizable. It can be isomerized to the *cis, trans*-isomer *via* the lactone, hence the *trans, cis*-configuration is excluded for it. Therefore, the first acid (II) should reasonably be concluded to be *cis-cis*, also by the expectation that it is formed by fission of an aromatic ring under a mild condition; then it will appear in a coiled phase with the orientation of *cis, s-cis, cis* at the first step.

The conclusions on the configurations of each acid are in complete agreement with spectral data.

The ultra-violet light absorption data of the isomeric α-methylmuconic acids and their esters are collected in TABLE I, together with those of some homologous isomers. Positions of the absorption maximum of each isomer are practically the same. There is no significant *cis*-shift, but the extinction coefficients show a significant difference in every case; the coefficient of *cis, cis*-isomer is the lowest and each *cis*→*trans* inversion increases the intensity. The validity of these criteria was also discussed in the series of α-methyl-$\alpha\beta$-unsaturated acids, reported in the previous paper of the authors[1a].

TABLE I

ULTRA-VIOLET LIGHT ABSORPTION OF ISOMERIC α-METHYLMUCONIC ACIDS IN COMPARISON WITH SOME HOMOLOGUES.

	cis-cis		*cis-trans*		*trans-trans*	
	λ max. (mμ)	ε	λ max. (mμ)	ε	λ max. (mμ)	ε
α-Methyl-muconic acid	269	20,700	269	25,700	273	29,700
methyl ester	270	24,800	272	27,600	275	31,100
Muconic acid[4]	258	17,000	259	25,600	259	29,100
methyl ester	259	26,400	260	29,800	259	36,700
$\alpha\alpha'$-Dimethyl-muconic acid[5]	281	20,400	280	25,500	282	31,450
methyl ester	280	24,750	280	31,600	282	33,300
β-Methyl-muconic acid[6]	—	—	265	19,000	265	22,400
methyl ester	—	—	265	22,100	266	28,400

The *cis, cis*-acid has a maximum extinction coefficient of 20,700 at 269 mμ, the *cis, trans*-acid, 25,700 at 269 mμ, and the *trans,trans*-acid, 29,700 at 273mμ. The methyl esters have the values of 24,800 at 270 mμ, 27,600 at 272 mμ and 31,100 at 275 mμ, respectively. These values unequivocally agreed with the expected values for each configuration.

In TABLE II are tabulated the characteristic bands in infra-red absorption spectra of the isomers.

TABLE II

CHARACTERISTIC BANDS IN THE INFRA-RED ABSORPTION SPECTRA OF THE ISOMERIC α-METHYLMUCONIC ACIDS (cm^{-1})

cis-cis	*cis-trans*	*trans-trans*
—	995	988
839	822	820
736	—	—

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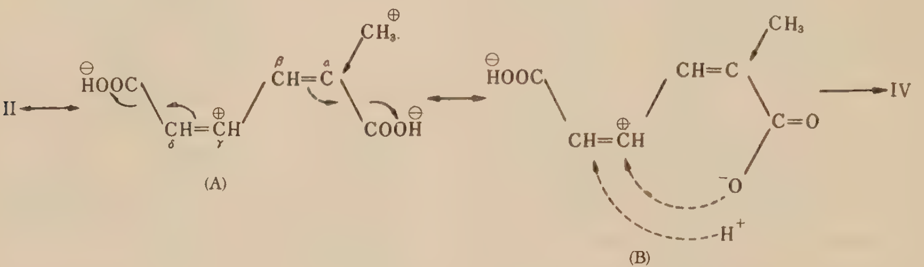
[4] J. A. Elvidge, R. P. Linstead, P. Sims and B. A. Orkin, *J. Chem. Soc.*, **1950**, 2235.

[5] J. A. Elvidge, R. P. Linstead and J. F. Smith, *J. Chem. Soc.*, **1952**, 1026.

[6] J. A. Elvidge, R. P. Linstead and P. Sims, *J. Chem. Soc.*, **1951**, 3386.

The uniformity of the lactonization of the *cis, cis*-isomer (II) may be interpreted as follows:



In the starting acid (II), the electrons are oriented as indicated in A. The electron attractive effect of the carboxyl group, attached to the δ-carbon atom, induces the polarization of the adjascent double bond producing a positive charge on the γ-carbon atom. The same effect of the carboxyl group, attached to the α-carbon atom, also produces a positive charge on the β-carbon atom, which is much more weakened by the electron releasing effect of the α-methyl substituent, hence, the positive charge on the β-carbon atom is weaker than that on the γ-carbon atom. Consequently, the attack of the polarized α-carboxyl-oxygen to the γ-carbon atom is preferred and then, followed by elimination of the carboxyl hydrogen as a proton and concomitant addition of the proton to the δ-carbon atom, resulting in the formation of the only known lactonic acid (IV).

EXPERIMENTAL

Melting and boiling points were uncorrected. Ultraviolet light absorption were determined in ethanol on a Beckman DU quartz spectrophotometre. Infra-red absorptions were determined on a Shimadzu Model AR-275 double-beam recording spectrophotometre. Microanalyses were carried by the Microanalytical Division of Prof. Mitsui's Laboratory, to whom the the authors' thanks are due.

α-Methyl-*cis, cis*-muconic acid (II) A mixture of 73.5 g. (0.68 mole.) of *o*-cresol (I) and 790 ml. of 13% peracetic acid solution (1.36 mole. of peracetic acid) was cooled in ice for 24 hrs. and then kept cold for 1 or 2 weeks. After the duration, the crystalline precipitate was collected, washed with acetic acid and recrystallized from methanol by cooling with dry-ice, to give the pure α-methyl-*cis, cis*-muconic acid in prisms, m.p. 189–90° (this m.p. was taken from a bath at 160°, with the temperature rising 10° per minute, since it varied with the rate of heating), λ max. 269 mμ ε 20,700 (*Anal.* Found: C, 53.73; H, 5.26. Calcd. for $C_7H_8O_4$: C, 53.84; H, 5.16), yield 12.3 g. (11.3%).

Quantitative hydrogenation:—A 0.103 g. quantity of the acid (II) in 60 ml. of methanol was hydrogenated over a platinum oxide catalyst (10 mg.) in a shaking apparatus and absorbed 36 ml. (at 20°) of hydrogen, equivalent to two double bonds. The reduction product was freed from the catalyst and the solvent, and recrystallized from benzene to give α-methyladipic acid (XVI), m.p. 58–9° (Mazza and DiMase[7] recorded m.p. 61°) (*Anal.* Found: C, 52.57; H, 7.45. Calcd. for $C_7H_{12}O_4$: C, 52.49; H, 7.55), also characterized by diamide, m.p. 185–6° (Bouveault and Locquin[8] recorded m.p. 186.5°), (*Anal.* Found: C, 53.06; H, 8.91; N, 17.79. Calcd. for $C_7H_{14}O_2N_2$: C, 53.14; H, 8.97; N, 17.71).

Methyl α-methyl-*cis, cis*-muconate (III) The *cis, cis*-acid was treated with ethereal diazomethane solution in the usual manner and the dimethyl ester was obtained in the yield almost quantitative. After recrystallization from light petroleum (b.p. 40–50°), it formed prisms, m.p. 35°, λ max. 270 mμ ε 24,800, (*Anal.* Found: C, 58.70; H, 6.80. Calcd. for $C_9H_{12}O_4$: C, 58.69; H, 6.57).

γ-Carboxymethyl-α-methyl-Δα-butenolide (IV) (**a**) A 3.62 g. quantity of the *cis, cis*-acid (II) was shaken with 36 ml. of cold 80% sulphuric acid for

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[7] F. P. Mazza and G. DiMase, *Gazz. chim. ital.*, **57**, 300 (1927).

[8] L. Bouveault and R. Locquin, *Compt. rend.*, **146**, 138 (1908); *Bull. Soc. Chem. France*, [4] **3**, 451 (1908).

1 hr. After 24 hrs., the reaction mixture was poured onto ice and the bulk of the acid was neutralized with an aqueous ammonia (acid to Congo-red). The solution was thoroughly extracted with ether. After removal of ether, the product was recrystallized from chloroform to yield the lactonic acid (IV) in prisms, m.p. 105–6° (*Anal.* Found: C, 53.60; H, 5.34. Calcd. for $C_7H_8O_4$: C, 53.84; H, 5.16), yield 3.08 g.

(b) A 0.75 g. quantity of the *cis, cis*-acid (II) was heated with water under reflux for 1 hr. The reaction mixture was dried up on a water bath and the residue was recrystallized from ethyl acetate to yield γ-carboxymethyl-α-methyl-Δα-butenolide, m.p. 105° (*Anal.* Found: C, 53.88; H, 5.03. Calcd. for $C_7H_8O_4$: C, 53.84; H, 5.16), the mixed m.p. with the product of the procedure (a) was 105°, yield 0.60 g.

γ-Carbomethoxymethyl-α-methyl-Δα-butenolide (V) To the lactonic acid (IV; 9.21 g., 0.059 mole.) in ether was added an ethereal solution of diazomethane (0.065 mole.) under cooling and well stirring, the product then distilled under reduced pressure to give the lactonic ester, b.p. 150–1°/9 mm., n_D^{20} 1.4741. (9.28 g.).

γ-Carbomethoxymethyl-α-methylbutanolide (VI) A 1.314 g. quantity (0.0077 mole.) of the unsaturated lactonic ester (V) in 50 ml. of methanol was hydrogenated over platinum catalyst and it took up 218 ml. (at 20°) of hydrogen, equivalent to 1.1 mole. The reduction product was freed from the catalyst and the solvent, and distilled to give the saturated lactonic ester, b.p. 136–9°/5 mm.; it crystallized from ether-light petroleum in laths, m.p. 45–6° (*Anal.* Found: C, 55.73; H, 6.98. Calcd. for $C_8H_{12}O_4$: C, 55.80; H, 7.03).

(δ)-Methyl (α)-hydrogen α-methyl-Δγ-dihydromuconate (VII) To 0.74 g. (0.0043 mole.) of the saturated lactonic ester (VI) in 8 ml. of methanol was added 1.64 ml. of methanolic sodium methoxide (2.62 N, 0.0043 mole.) at room temperature. After 15 min., the reaction mixture was evaporated under reduced pressure and then, was added 6 ml. of water and it was acidified with dilute hydrochloric acid. An oily substance separated was extracted with ether. The solvent was removed and the residual product was completely dried over paraffin *in vacuo*, n_D^{22} 1.4642, yield 1.20 g.

Ozonization:—A 1.7 g. quantity of the half-ester in chloroform was treated with an excess of ozone at 0°. The solvent was removed *in vacuo*, and the remaining ozonide was decomposed with water on a water bath for 10–15 min. and extracted with ether. The ethereal solution was distilled under reduced pressure to give methyl hydrogen oxalate (IX), b.p. 110–4°/8 mm. (R. Anschütz[9], b.p. 108–9°/12 mm.), yield 0.75 g. This half-ester was readily hydrolyzed to oxalic acid, m.p. 186°, not depressed by admixture with an authentic specimen. The residue of the distillation was recrystallized from ethyl acetate, to yield methylsuccinic acid (X), m.p. 106°, not depressed by mixed melting point comparison with an authentic specimen[10], yield 0.9 g. This acid was also characterized by di-*p*-phenylphenacyl ester, m.p. 179° (*Anal.* Found: C, 75.92; H, 5.54. Calcd. for $C_{33}H_{28}O_6$: C, 76.14; H, 5.42).

α-Methyl-Δγ-dihydromuconic acid (VIII) A 1.17g. quantity of the half-ester (VII) was warmed on a steam bath with 12.5 ml. of 1N sodium hydroxide for 1 hr., then, the reaction mixture was acidified with dilute hydrochloric acid, and extracted with ether. After removal of ether, the acid was recrystallized from benzene in needles, m.p. 121–2° (*Anal.* Found: C, 53.38; H, 6.44. Calcd. for $C_7H_{10}O_4$: C, 53.16; H, 6.37), yield 0.64 g.

(δ)-Methyl (α)-hydrogen α-methyl-*cis, trans*-muconate (XI) To the unsaturated lactonic ester (V; 5.09 g., 0.0299 mole.) in 50 ml. of methanol, was added 12.5ml. of methanolic sodium methoxide (2.39 N). After 15 min., the solution was evaporated under reduced pressure. Water (50 ml.) was added and the solution was acidified with hydrochloric acid, whereupon methyl hydrogen α-methyl-*cis, trans*-muconate separated, which crystallized from benzene in prismatic needles, m.p. 121°, λ max. 275 mμ ε 22,000 (*Anal.* Found: C, 56.53; H, 5.98. Calcd. for $C_8H_{10}O_4$: C, 56.46; H, 5.92) yield 4.60 g.

Reconversion into the lactonic ester:—When the half-ester (0.27 g.) was heated at 170–5° under reduced pressure, a liquid distillate was obtained. After redistillation it gave the unsaturated lactonic ester (V), b.p. 150–5°/11 mm., n_D^{17} 1.4820 (yield 0.18 g.), which was identified by the infra-red absorption spectrum.

α-Methyl-*cis, trans*-muconic acid (XII) A 1.50 g. quantity of the preceding half-ester (XI) was warmed with 17.6 ml. of 1N sodium hydroxide on a steam bath for 1 hr. After the duration, the solution was acidified with hydrochloric acid, the separated crystal was recrystallized from methanol and from ethyl acetate to yield the *cis, trans*-acid in plates, m.p.

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[9] R. Anschütz, *Ann.*, **254**, 1 (1889).

[10] *Org. Synth.*, *Coll. Vol.* III, p. 615.

172°, λ max. 269 mμ ε 25,700 (*Anal.* Found: C, 53.87; H, 5.23. Calcd. for $C_7H_8O_4$: C, 53.84; H, 5.16) yield 1.35 g.

Hydrogenation:—The hydrogenation of the *cis, trans*-acid (XII) over a platinum catalyst gave α-methyladipic acid (XVI), having m.p. and mixed m.p. 58–9°.

Methyl α-methyl-*cis, trans*-muconate (XIII) Treatment of methyl hydrogen α-methyl-*cis, trans*-muconate (XI) with ethereal diazomethane, and evaporation of the solution afforded the dimethyl ester which, after recrystallization from methanol, formed prisms, m.p. 60°, λ max. 272 mμ ε 27,600 (*Anal.* Found: C, 58.84; H, 6.70. Calcd. for $C_9H_{12}O_4$: C, 58.69; H, 6.57). The same dimethyl ester was also obtained by the same procedure from the *cis, trans*-acid (XII).

α-Methyl-*trans, trans*-muconic acid (XIV) (a) α-Methyl-*cis, cis*-muconic acid (II, 0.51 g.) was heated with 30 ml. of 20% aqueous sodium hydroxide for 4 hrs. The solution was cooled and acidified with dilute sulphuric acid. The separated precipitate was recrystallized from methanol to yield the *trans, trans*-acid in prisms, m.p. 273° (R. Kuhn and J. Michel[2] recorded m.p. 276°), λ max. 273 mμ ε 29,700 (*Anal.* Found: C, 54.00; H, 5.29. Calcd. for $C_7H_8O_4$: C, 53.84; H, 5.16), yield 0.45 g.

(b) α-Methyl-*cis, trans*-muconic acid (XII) gave the same acid exactly by the same procedures as mentioned above.

Hydrogenation of the *trans, trans*-acid gave α-methyladipic acid (XVI), as was the case with the *cis, cis*- and *cis, trans*-acids.

Methyl α-methyl-*trans, trans*-muconate (XV) The esterification of the *trans, trans*-acid with diazomethane gave the dimethyl ester in plates (from methanol), m.p. 55.5°, λ max. 275 mμ ε 31,100 (Harris and Binns[3] reported m.p. 46–7°, λ max. 276 mμ log ε 4.17), (*Anal.* Found: C, 58.51; H, 6.87. Calcd. for $C_9H_{12}O_4$: C, 58.69; H, 6.57).

The authors are indebted to Prof. S. Takei for his helpful advice in this work and also to Dr. M. Maesawa of Central Research Laboratory of Osaka Coal Gas Company for his kind gift of *o*-cresol.

[Bull. Agr. Chem. Soc. Japan, Vol. 22, No. 3, p. 168～174, 1958]

Amylase Formation by Lysozyme Lysate of *Bac. subtilis*

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Received January 23, 1958

Bacterial amylase was formed by a subcellular system that was obtained by treating cells of an amylase-producing bacteria with lysozyme. However, the response of enzyme-forming ability of the lysate to various carbon and nitrogen sources was found to be significantly different from that of vegetative cells. The present paper describes some properties of the lysate and the effects of carbon and nitrogen sources on enzyme formation by the lysate, with a comparison of those by the vegetative cells.

In the previous studies[1] on formation of bacterial amylase, it has been found that a certain amount of amylase was secreted when a suspension of washed cells of *Bac. amyloliquefaciens* Fukumoto was shaken aerobically without any addition of nitrogen source, in the presence of phosphate and a sugar or an organic acid as a carbon source. It has also been found that the amylase formation caused by the suspension of washed cells was inhibited by the addition of chemical such as chloramphenicol, 8-azaguanine or several cell respiration inhibitors such as monoiodoacetic acid, potassium cyanide, etc.[2,3]

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[1] J. Fukumoto and T. Yamamoto, *Symposia Enz. Chem. (Japan)*, 7, 3 (1951).

Gale and Folkes[4) in 1955 reported that a cell-free preparation obtained from *Staphylococcus aureus* exhibited the activities of forming certain kinds of enzymes. Thenceforth, several papers[5,6,7) on the application of such a cell-free system in the investigation of enzyme formation have been published. Recently, Nomura and his coworker[8] have reported on amylase formation by a subcellular preparation which was obtained by treating cells of *Bac. subtilis* with lysozyme.

The lysozyme lysate of *Bac. amyloliquefaciens*, amylase formation of which bacteria having been studied by the authors using its washed cells, was also found to form the enzyme. However, in sharp contrast with a suspension of washed cells, enzyme formation by the lysate was observed only when some organic acids were supplied as the carbon source to the system. Furthermore, this enzyme formation was found not to be affected by the addition of nitrogen source such as amino acid mixture which when added to a suspension of washed cells has been known to greatly stimulate the enzyme formation. These facts themselves seems to give the evidence that the enzyme formation observed in the experiment was surely dependent upon a non-vegetative cell system. These facts also appear to serve as a criterion in investigating whether the lysate used in the experiment is free from vegetative cells or not. This paper deals with the amylase formation by a subcellular system (lysozyme lysate) of *Bac. amyloliquefaciens* Fukumoto comparing that with a suspension of washed cells of the microorganism.

MATERIALS AND METHODS

As reported previously, *Bac. amyloliquefaciens* Fukumoto, a species of strain of *Bac. subtilis,* was used throughout the experiment. The organism was grown by surface culture at 37°C for 40 hrs. on a medium containing 5% soybean-cake extract, 3.5% dextrin and 1.2% $(NH_4)_2HPO_4$ (pH 7.0). After cultivation, the cells were harvested and washed five times with distilled water using a centrifuge. The washed cells were finally suspended in 0.1M phosphate buffer of pH 7.0 (about 10 mg dry matter per ml).

A lysozyme lysate of the bacteria was prepared as follows: 5 mg of crystalline lysozyme was added to 50 ml of the suspension of washed cells and the mixture was incubated at 30°C for 30 to 40 min.

Amylase activity was determined by a modification of the Lintner-Sollied method[9). One-unit of activity of this method was found to be roughly equivalent to four units of the method of Smith and Roe[10). The degree of cell lysis was followed by measuring the change in optical density at 600 mμ using a cylindrical holder, 1.7 cm in diameter, of an Erma spectrophotometer. Oxygen consumption was determined by conventional manometric technique at 36°C.

EXPERIMENTAL RESULTS

1. Some Properties of the Lysozyme Lysate of the Bacteria.

The lysate obtained by the method described above revealed to contain only disrupted cells under microscopic observation (Fig. 1). The debris found in the lysate were not precipitated by centrifugation at 800 ×g for 10min., while the intact cells were completely precipitated. However, the debris were precipitated at 2600×g for 10 min., retaining about 50% of nitro-

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2) J. Fukumoto, T. Yamamoto and D. Tsuru, *Symposia Enz. Chem. (Japan)*, **12**, 259 (1957).

3) J. Fukumoto, T. Yamamoto and D. Tsuru, *Nature*, **180**, 438 (1957).

4) E. F. Gale and J. P. Folkes, *Biochem. J.*, **59**, 675 (1955).

5) K. McQuillen, *Biochim. Biophys. Acta*, **18**, 458 (1955).

6) O. E. Landman and S. Spiegelman, *Proc. Nati. Acad. Sci.*, **41**, 698 (1955).

7) F. B. Straub, A. Ullman and G. Acs, *Biochim. Biophys. Acta*, **18**, 439 (1955).

8) M. Nomura, B. Maruo and S. Akabari, *J. Biochem.*, **43**, 251 (1956).

* Soybean-cake extract was prepared as follows: One-part of the cake was bolied with twenty-parts of 0.2% NaOH for 1 hr. The mixture was then neutralized and filtered, the filtrate used as the soybean-cake extract.

9) J. Fukumoto and T. Yamamoto, *J. Agr. Chem. Soc. (Japan)*, **31**, 421 (1957).

10) B. W. Smith and J. H. Roe. *J. Biol. Chem*, **179**, 53 (1949).

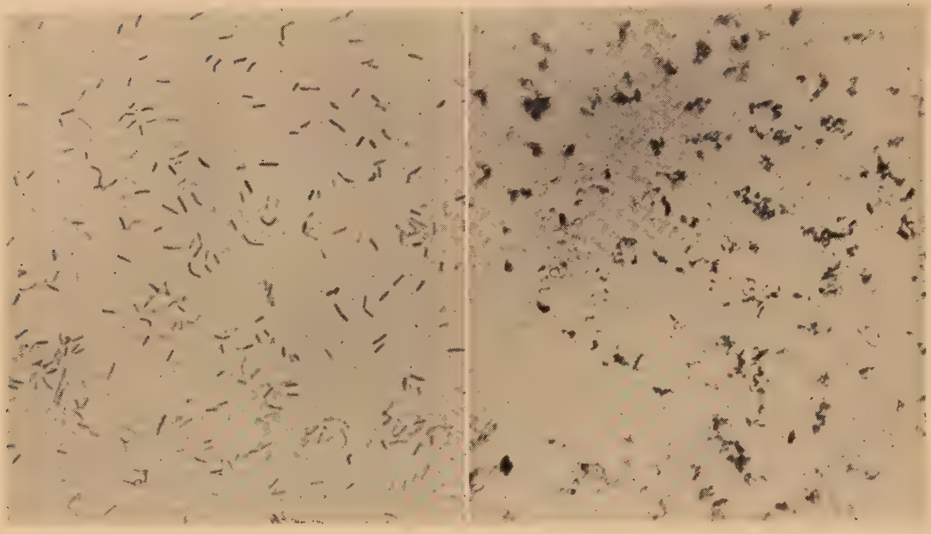
(a) (b)

FIG. 1. Micrographs of *Bac. amyloliquefaciens* Fukumoto:

Preparations were stained with 0.5% methylene blue.
a) intact cells, b) disrupted cells found in the lysate.

TABLE I. RELEASE OF NITROGENOUS COMPOUNDS AND CARBOHYDRATES FROM THE CELLS BY LYSOZYME TREATMENT.

	Supernatant liquid*	Precipitated fraction*
Total nitrogen, %	52.9	46.9
Total sugar as glucose after hydrolysis, %	52.5	47.2

* Precipitation procedure is described in the text.

gen of the intact cells (Table I) and both activities of amylase formation and respiration under the condition described later.

The lysate, optical density of which had been reduced to about 20% of that of the original cell suspension, showed almost no change in turbidity during the subsequent aerobic incubation of three hrs., even in the presence of both carbon and nitrogen source, as shown in Fig. 2.

The lysate did not consume oxygen in the presence of ordinary sugar, but oxygen was consumed when organic acid such as pyruvic, succinic or fumaric acid was supplied (Fig. 3). The activity of oxygen consumption of the system was only about one-third of that of the vegetative cell, using pyruvic acid as a carbon source. The system also consumed oxygen in the presence of β-glycerophosphate or glucose-6-

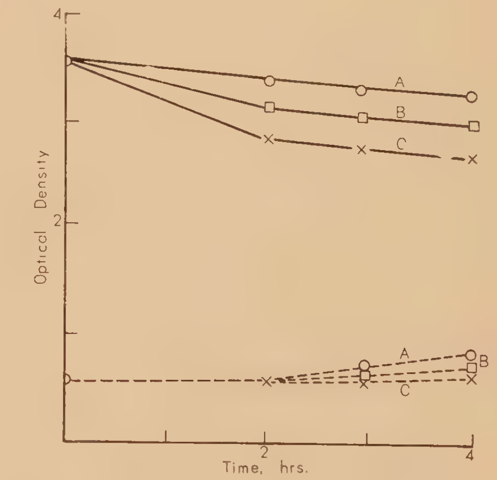

FIG. 2. Changes in Turbidity of Washed Cell Suspension and Lysate in the Course of Aerobic Incubation: (50 mg of vegetative cells or lysate, 0.5% carbon source, M/10 phosphate buffer, pH 7.0, total volume 12 ml).

A, galactose; B, Na-pyruvate; C, no carbon source; Solid and broken lines indicate changes in turbidity of washed cell suspensions and lysate, respectively.

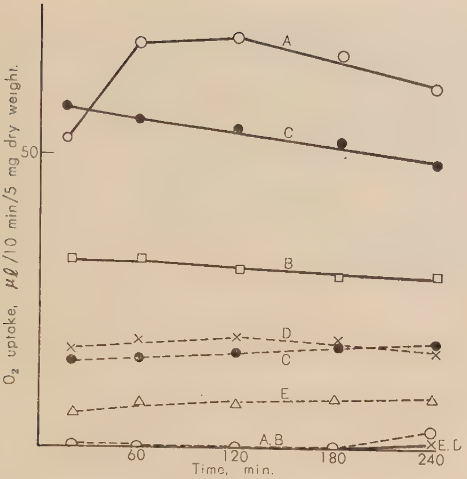

FIG. 3. Effects of Carbon Sources on Respiration of Vegetative Cells and Lysate: (50 mg of vegetative cells or lysate, 0.5% carbon source, M/10 phosphate buffer, pH 7.0 total volume 2 ml, 36°C).

A, glucose; B, galactose; C, Na-pyruvate; D, β-glycerophosphate; E, glucose-6-phosphate; solid and broken lines indicate the respiration of vegetative cells and lysate, respectively.

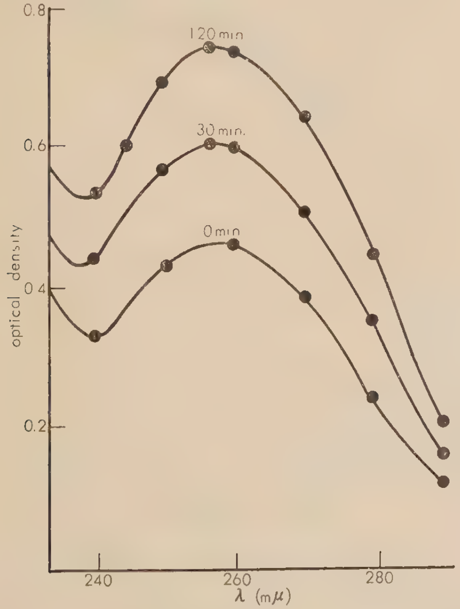

FIG. 4. Decomposition of Nucleic Acid appeared in the Supernatant of the lysate.

The lysate prepared by the method described in the text were centrifuged at 4000×g for 10 min., and the supernatant was incubated at 37°C. Aliquots were removed at the intervals indicated and mixed with seven volumes of the cold precipitating reagent*. The mixtures were then centrifuged and the ultraviolet absorption spectra of the supernatants were measured.

phosphate, in contrast to the vegetative cells which did not exhibit the activity on these substrates.

During the treatment of the bacterial cells with lysozyme, a considerable amount of nucleic acid appeared in the supernatant liquid of the lysate and was decomposed into substances which were not precipitated by the addition of nucleic acid-precipitating reagent[11]. The decomposition of nucleic acids was found to continue, even after removal of the cell debris, as seen in Fig. 4.

2. Amylase Formation by the Subcellular System.

As shown in Fig. 5, amylase formation by the lysate system was observed only in the presence of certain kinds of organic acids. The ability of the system to form amylase was about one-third of that of the vegetative cells, even in the presence of pyruvic acid with which oxygen consumption by the lysate was shown to be most active.

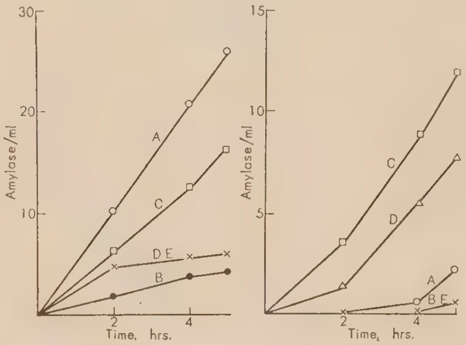

FIG. 5. Effects of Carbon Sources on Amylase Formation by Vegetative Cells and Lysate: (The experimental conditions were the same as those in Fig. 2).

A, galactose; B, glucose; C, Na-pyruvate; D, Na-citrate; E, no carbon source.

Amylase formation by the lysate as well as by the suspension of washed cells was found to be severely inhibited by addition of chloramphenicol (10 γ/ml), monoiodoacetic acid (M/2000) or potassium cyanide (M/200), but not influenced by penicillin, as shown in Table II.

Supply of nitrogen source such as amino acid mixture or polypeptone to the suspension of washed cells, together with a suitable carbon source, greatly increased the amylase formation, as it has been previously

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*11) R. Schucher and L. E. Hokin, *J. Biol. Chem.*, **210**, 551 (1954).

TABLE II. INFLUENCE OF SOME CHEMICALS ON AMYLASE FORMATION BY VEGETATIVE CELLS AND LYSATE

(The experimental conditions were the same as those in Fig. 2, except that 0.5% of Na-pyruvate was used as the carbon source.)

Additions	Concentration	Amylase per ml	
		Vegetative cells	Lysate
None	—	34.0	13.0
CH_2ICOOH	M/2000	2.8	1.9
〃	M/1000	1.8	2.0
KCN	M/400	18.0	7.2
〃	M/200	9.0	4.6
Chloramphenicol	10γ/ml	2.0	1.1
〃	50γ/ml	1.0	1.0
Penicillin G	0.5 mg/ml	36.5	13.6
〃	1.0 mg/ml	34.8	13.5

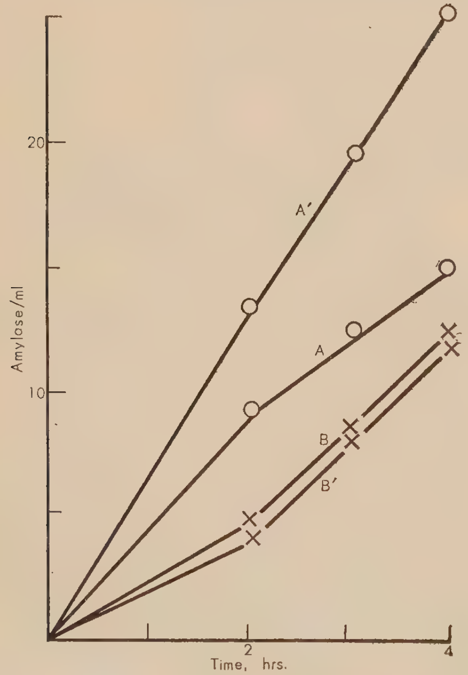

FIG. 6. Effect of Amino Acids Mixture* on Amylase Formation by Vegetative Cells and Lysate: (The experimental conditions were the same as those in Fig. 5, except that 0.4% Na-pyruvate was used as the carbon source).

A, vegetative cells; B, lysate; A′, A+amino acids mixture; B′, B+amino acids mixture.

* Contained 1.5 mg each of glycine, alanine, glutamic acid, aspartic acid, methionine, phenylalanine and 1.0 mg each of lysine, leucine, tryptophan, tyrosine oxyproline, histidine, serine and threonine per ml. Added one ml of this solution to 9 ml of incubation mixture.

reported[12]. However, the supply of nitrogen source to the lysate was found to give no effect on the enzyme formation, as shown in Fig. 6.

Amylase formation by the vegetative cells was inhibited by addition of 8-azaguanine (M/500), although the inhibition degree varied according to the type of carbon source employed[3]. However, the enzyme formation by the lysate was not influenced by the reagent (Fig. 7).

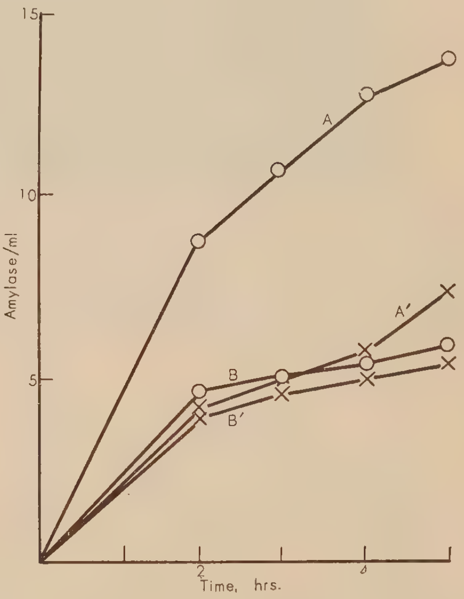

FIG. 7. Influence of 8-azaguanine on Amylase Formation by Vegetative Cells and Lysate: (The experimental conditions were the same as those in Fig. 6).

A, vegetative cells; B, lysate; A′, A+M/300 8-azaguanine; B′, B+M/300 8-azaguanine.

TABLE III. INFLUENCE OF RIBONUCLEASE ON AMYLASE FORMATION BY VEGETATIVE CELLS AND LYSATE

(The experimental conditions were the same as those in Table 2, except that the amount of cells used were 40 mg.)

	Vegetative cells			Lysate		
Ribonuclease, γ/ml	—	20	100	—	20	100
Amylase/ml	18.0	17.2	20.0	11.3	13.2	5.0

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[12] J. Fukumoto, T. Yamamoto and D. Tsuru, *J. Agr. Chem. Soc.* (*Japan*), **31**, 724 (1957).

On the other hand, amylase formation by the lysate was inhibited to some extent by addition of crystalline ribonuclease prepared from bovine pancreas, while the enzyme formation by the vegetative cells was not affected by such treatment, as shown in Table III.

DISCUSSION

In contrast with the vegetative cells, the lysate was found to consume oxygen and form amylase only in the presence of certain kinds of organic acids. From this result it is certain that the system contained almost no intact cell and consumption of oxygen and formation of amylase were completely due to the disrupted-cell system.

The fact that the lysate consumed no oxygen in the presence of ordinary sugars as a carbon source seems to indicate that the system had been devoid of the function of phosphorylation of sugar. As seen from Table II, both activities of respiration and amylase formation of the lysate system were inhibited by addition of monoiodoacetic acid, potassium cyanide or chloramphenicol as those in vegetative cells. This leads to the suggestion that for the enzyme formation by this system, the supply of energy is also indispensable and thus the mechanism of amylase formation or, at least, its appearance as an active enzyme is not so simple as it has been found in the activation of trypsinogen to trypsin.

As shown in Fig. 6, the supply of nitrogen source to the lysate did not have any effect on amylase formation. This result seems to present an indication that the enzyme formation by the lysate takes place using only a certain nitrogen material which might be a precursor of the enzyme.

In the experiment shown in Fig. 7, 8-azaguanine did not change the activity of amylase formation in the lysate, whereas it inhibited the enzyme formation by vegetative cells. This non-effectiveness of 8-azaguanine for the lysate system may be considered to be due to the reverse effect of some decomposition products of nucleic acids (Fig. 4). However, it may be due to another reason that amylase formation by the lysate occurs without any new synthesis of nucleic acid. Investigation to ascertain these questions is now progress.

In the experiments for the preparation of the lysozyme lysate, it sometimes occurred to result in a remarkable loss in both activities of enzyme formation and oxygen consumption. The reason of a simultaneous loss in both activities observed in the experiment and the method to safely obtain the preparation are also under investigation.

SUMMARY

By treating the suspension of washed cells of *Bac. amyloliquefaciens* with lysozyme, a lysate was prepared under the described condition and some properties of the lysate were investigated in regard to amylase formation, comparing with those of vegetative cells. The results obtained are as follows:

1. The lysate consumed oxygen and formed amylase only when organic acid such as pyruvic, succinic, fumaric or citric acid was supplied.
2. Both activities of amylase formation and respiration of the lysate were found in the cell debris which were precipitated by centrifuging at 2600×g for 10 min., retaining about 50% of nitrogen of the intact cells.
3. Both the activities of the lysate, as those in vegetative cells, were remarkably inhibited by addition of monoidoacetic acid (M/2000), potassium cyanide (M/200) and chloramphenicol (10γ/ml).
4. Amylase formation by the lysate was not favoured by addition of an additional nitrogen source such as amino acid mixture or polypeptone.
5. The enzyme formation by the lysate was not influenced by addition of 8-azaguanine whereas in case of vegetative cells was severely inhibited by the base analogue.
6. By addition of crystalline ribonuclease,

amylase formation of the lysate was considerably inhibited while that of the vegetative cells not affected.

The authors are indebted to Mr. S. Naono of Osaka University and to Mr. H. Yamada of Kyoto University, for kindly supplying the ribonuclease and glucose-6-phosphate used in this work.

[Bull. Agr. Chem. Soc. Japan, Vol. 22, No. 3, p. 174~176, 1958]

Shikimic Acid in the Tobacco Leaf

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Received January 29, 1958

Shikimic acid in flue-cured tobacco leaf was identified and determined by paper chromatography. The presence of shikimic acid in the flue-cured leaf was confirmed, although the content was very small, 0.02 per cent of dry matter.

In a previous paper,1) the author has reported that chlorogenic acid in tobacco leaves is newly formed in considerably great quantities during flue-curing and it seems reasonable to conclude that the aromatic ring, caffeic acid, is synthesized by a route through sedoheptulose, that is, shikimic acid. Recently, Geisman has reported2) that caffeic acid in the tobacco leaves fed in the seedling stage with labeled precursor, phenylalanine-2-C^{14}, derive directly and specifically its whole molecule from phenylalanine, and it is concluded that in higher plants such as in microorganisms, aromatic rings are made by two pathways one through shikimic acid and the other through head to tail linkage of acetic units.

The author has attempted to identify and determine shikimic acid, that seemed to be a normal intermediate in the route of aromatic ring formation in the tobacco leaf. In a recent survey of organic acids in fruits,3-5) grass,6) and other plant tissues,7-10) shikimic acid has been found in a considerable amount. However, in the tobacco leaf shikimic acid has not been found up to the present. In this paper, shikimic acid in the tobacco leaf was identified and determined by paper chromatography11) and the spectrophotometric method described by Yoshida and Hasegawa.10)

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1) M. Nagasawa, This Bulletin, **22**, 21 (1958).

2) T. A. Geisman and T. Swain, *Chem. & Ind.*, **1957** 984.

3) G. C. Whiting, *Nature*, **179**, 531 (1957).

4) D. E. Hathway, *Biochem. J.*, **63**, 380 (1956).

5) A. C. Hulme, *Nature*, **178**, 991 (1956).

6) A. Richardson and A. C. Hulme, *Nature*, **175**, 43 (1955).

7) M. Hasegawa, S. Yoshida and T. Nakagawa, *Kagaku (Japan)*, **24**, 421 (1954).

8) M. Hasegawa, T. Nakagawa and S. Yoshida, *Nippon Ringaku Kaishi (Japan)*, **39**, 159 (1957).

9) S. Hattori, S. Yoshida and M. Hasegawa, *Physiol. Plant.*, **7**, 283 (1954).

10) S. Yoshida and M. Hasegawa, *Arch. Biochem. Biophys.*, **70**, 377 (1957).

11) E. F. L. J. Anet and T. M. Reynolds, *Nature*, **172**, 1188 (1953); *Aust. J. Chem.* **24**, 489 (1955).

EXPERIMENTAL

Preparation of Extracts. One hundred grams of moisture-free material of Japanese flue-cured tobacco, produced in Kagoshima district, 1956 crop (Rapper, 2 grade), which had been ground in a mill fine enough to pass a 1-mm sieve, were boiled for one hour under a reflux condenser with 1500 ml of 80 per cent methanol. The mixture was filtered, and the residual tobacco was boiled again for one hour with 1000 ml of 80 per cent methanol. The mixture was subsequently filtered and washed with 80 per cent methanol. The combined filtrate and washings were concentrated under reduced pressure, and then 1000ml of water was added to the residue and filtered. The filtrate was first passed through a column of Dowex 50X4 resin in the hydrogen form. The collected effluent was passed through a column of Amberlite IR4B resin in the hydroxyl form. In order to recover the acids from the resin a column of IR4B was eluted with 1N aqueous ammonia. The emergent solution below pH 10 was collected, and then treated with Dowex 50X4 resin in the hydrogen form. The solution containing organic acid was concentrated under reduced pressure and transferred quantitatively to a 25 ml volumetric flask and made to volume with water.

Qualitative Separation and Identification. The methanolic extracts were investigated by paper chromatography using the following two solvents: (I) methylethylketone-cineole-formic acid-water (50:50:20:16),[11] (II) amylalcohol-5*N* formic acid (1 : 1).[12] Shikimic acid was observed in both solvents with R_F values, 0.15 and 0.20 respectively, and gave a red color on spraying periodate and anilin reagents described by Yoshida and Hasegawa.[10] Moreover, shikimic acid gave a greenish yellow color on spraying with periodate followed by sodium nitroprusside and piperazine described by Cartwright and Roberts.[13] Identical R_F values (alone and mixed) and the same colors were given for the acid spot detected and an authentic shikimic acid in the above two solvent systems and reagents.

Quinic acid in the tobacco extracts was separated and observed with R_F values in both solvents, 0.09 and 0.08 respectively.

Quantitative Assay. A spectrophotometric determination of shikimic acid[10] was applied to the aqueous eluate of paper chromatogram (I). One hundred and forty microliters of the methanolic extracts (25 ml) were applied to Whatman No. 1 paper sheets. The organic rich phase of solvent (I), described above, was added to the trough and chromatograms were run descending-wise. After about eighteen hours, the strip was removed and dried in a forced-draft hot oven at 70° for one hour. The areas, detected by test-spots on both sides of paper sheets, were cut out and eluted with water. The eluate was transferred quantitatively to a 10 ml volumetric flask and made to volume with water. An aliquot of 2 ml of the eluate was used for the color reaction, based on the reaction of the periodate oxidation of shikimic acid with anilin to yield a Schiff base, and the other aliquot was used for a blank solution without aniline for zeroing the spectrophotometer.

Among the reagents, described by Yoshida and Hasegawa, ethylene glycol solution was modified as follows: thirty milliliters ethylene glycol is dissolved in 100 ml distilled water to obtain a final clear solution for measurement.

Optical density measurements were made in a Beckman B spectrophotometer at 510 mμ using a blank solution described above.

The recovery of shikimic acid from paper chromatogram resulted in 88 per cent.

RESULTS AND DISCUSSION

The color reactions of shikimic acid contained in the tobacco leaf were identified, although both colors were pale. A greenish yellow color based on the reaction of the periodate oxidation product of shikimic acid with sodium nitroprussid and piperazine was specific for quinic, shikimic and dehydroshikimic acids.[3,4] On the other hand, a red color on the reaction of the periodate oxidation product of shikimic acid with aniline was specific for shikimic acid, while other organic acids did not react under these conditions. Neither sugars nor amino acids gave inhibitory effects, except polyhydroxy compounds such as carbohydrates or polyols.[10] These observations give aid to identification. Moreover, the R_F values (alone and mixed) were identical with authentic shikimic acid by the two solvent systems. Therefore, the presence of shikimic acid in flue-cured tobacco leaf was confirmed, although the content was very small. Shikimic acid content found in the flue-cured tobacco leaf was 0.02 per cent to dry matter by spectrophotometric determination.

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[12] M. L. Buch, R. Montgomery and W. L. Porter, *Anal. Chem.*, **24**, 489 (1952).

[13] R.A. Cartwright and E.A.H. Roberts, *Chem. & Ind.*, **1955**, 230.

The results of this investigation may lead to some support of the presence of a direct oxidative pathway in the tobacco leaf and the fact that chlorogenic acid is formed during flue-curing through sedoheptulose as reported previously,[1] and is also in accordance witn Geisman's report[2] that in the tobacco plant there is a possibility of aromatic ring formation through shikimic acid.

Quinic acid, which formed chlorogenic acid with caffeic acid, is present in the tobacco leaf, and the determination of quinic acid is now in progress.

ACKNOWLEDGMENT

The author wishes to thank Mrs. Taeko Taguchi for her assistance in carrying out the determination of shikimic acid, and the author is very grateful to Mr. S. Yoshida, of the Botanical Institute, University of Tokyo, for his criticism.

[Bull. Agr. Chem. Soc. Japan, Vol. 22, No. 3, p. 176~185, 1958]

Taxonomical Study of Glutamic Acid Accumulating Bacteria, *Micrococcus glutamicus* nov. sp.

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Received December 19, 1957.

Taxonomical studies of a new group of strains of glutamics acid accumulating bacteria isolated in our laboratory were carried out. It was found that these strains belong to Genus *Micrococcus* because they are gram-positive, none-sporulating, and catalase possessing cocci. They are also pale yellowish, aerobic, and capable to reduce nitrate. Moreover, they neither utilize ammonium salt as a sole source of nitrogen in Hucker's medium nor liquefy gelatin. From these observations, it seems that they are related to *M. aurantiacus* and *M. epidermidis*. However, our strains are different from them in the manner of acid production in milk and lactose media, and also in pigmentation, serological reactions and glutamic acid accumation.

The name of *Micrococcus glutamicus* nov. sp. was, therefore proposed to these strains.

The phenomenon of cell elongation and characteristics of the pigments of these strains was also investigated.

A new group of strains of *Micrococcus* which accumulates a large quantity of glutamic acid in culture medium was isolated by one of the authors.[1] This article is concerned with the identification of these strains to belong to a new species of genus *Micrococcus* which we named *M. glutamicus* nov. sp.

EXPERIMENTAL METHODS

Diagnostic tests were carried out according to

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[1] Shukuo Kinoshita, Shigezo Udaka and Masakazu Shimonō: Presented at the meetıng of the Agricultural Chemical Society of Japan. April 9–11, 1957. Tokyo.

"Manual of methods for pure culture study of bacteria" 6th Ed. 1950, Society of American Bacteriologists, and referred to "Handbook of practical bacteriology" by Mackie & MacCartney 9th Ed. 1953, E. & S. Livingstone, Edinburgh and London, and "Saikingaku Jitsu-shū Teiyō 1951, Maruzen, Tokyo and others.

Temperature used for incubation of bacteria was generally 37°C in most cases except in the case of gelatin liquefaction tests.

Precipitation and agglutination tests with immune serum were carried out as follows.

a) Immune serum: The cells of our strain No. 534 were harvested by centrifugation after shaking culture in glucose nutrient medium at 28°C for 2 days, and washed three times with saline water, then resuspended in saline water containing merzoin (1 : 1000) and stored in an ice box until it was used for injection. This cell suspension was injected intra-venously to healthy male rabbits. One rabbit was injected with this cell suspension five times at intervals of 5 days, and does of cell suspension employed in each time were 1.0, 1.5, 1.5 and 2.0 mg (as dried-cell weight), respectively. Therefore, the total dosage used per one rabbit was 7.0 mg. A blood sample was isolated in an ice box. After heating at 56°C for 30 minutes, serum was added with 0.5% phenol and stored in an ice box prior to use.

b) Antigen for precipitation tests: The cells of each species of bacteria were harvested as shown in (a). After washing twice with saline water, they were resuspended in saline water at the level of 0.1 ml wet cells per 1 ml, then heated at 100°C for 120 minutes and centrifuged. The supernatants thus obtained, were used as antigens for precipitation tests.

c) Precipitation tests: According to the usual method, each of above mentioned antigens was diluted, then added into a small test tube containing 0.3ml of immune serum against our strain No. 534. After incubation for 2–18 hours at 37°C, the occurrence of precipitation of serum was observed.

d) Slide agglutination reaction: The cells of each species grown on nutrient slanted agar for 24 hours were suspended in saline water and mixed with the above immune serum on slides according to the usual method, and the agglutination of the cells was tested. The immune serum was diluted if required and saline water was used as the control.

EXPERIMENTAL RESULTS

I. Decision of family

Our strains are Gram-positive, non sporulating, and catalase positive cocci. Therefore, they belong to Family V. Micrococcaseae described in Bergey's Manual 6th Ed..

Our strains have a rather ellipsoidal form. If our strains are regarded as rods, then there is a possibility that they belong to Family VII. *Corynebactreiaceae*. However, members of this family are usualy pathogenic and all species described in this family are not in accord with our strains. In Bergey's Manual 6th Ed., the groups which are not included in Family I to XI were included in Family XII, *Bacteriaceae*. (but the family name *Bacteriaceae* was rejected in 1953–1954, together with the genus name *Bacterium*.[2] If our strains are to be regarded as short rods, *Bacterium mutabile* and *Bacterium qualis* would be comparatively related with our strains, but the former is different from our strains in its gelatin liquefying ability and the latter is different in acid production from sugar, reaction with litmus milk and its branched pleomorphic form.

II. Decision of genus

In *Micrococcaceae*, there are 3 genera, i. e., *Micrococcus*, *Gaffkya*, and *Sarcina* and 2 appendicies, i. e., *Methanococcus*, and *Pediococcus*. *Methanococcus* contains only one species and is anaerobic. Species of *Pediococcus* do not reduce nitrate. According to Shaw, Stit and Cowan[3] it does not seem proper to differentiate *Gaffkya*, *Sarcina* and *Micrococcus*. According to the descriptions of Bergey's Manual, *Gaffkya* is parasite and contains 2 species. One species is a strict anaerobe and the other which does not reduce nitrate, is a facultative aerobe. Consequently, our strains cannot be included in the above 3 genera, i. e., *Methanococcus*, *Pediococcus* and *Gaffkya*. In *Sarcina*,

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[2] R. E. Buchanan, *Ann. Rev. of Microbiol.*, 9, 1 (1955).

[3] C. Shaw, J. M. Stit and S. T. Cowan, *J. Gen. Microbiol.*, 5 1010 (1951).

"cells occur in regular packets." Our strains sometimes form tetrad but do not produce packet. In case of *Micrococcus*, following descriptions can be seen in Bergey's Manual: "Cells occur in plates, groups or in irregular packets and masses, never in chains. Pigment when present is yellow, orange or red. Gram-positive to Gram-negative." So, our strains conform to these description. Winslow, G.E. and Winslow, A. R.[4] stated that the packet forming character is not stable and differentiation from the genus *Sarcina* is difficult. Shaw et al[3] and Abd-El-Malek & Gibson[5] held the same opinion. Furthermore, 9 species described in Bergey's Manual, all differ from our strains. So, we concluded that our strains belong to the genus *Micrococcus*. According to V. B. D. Skerman's[6] "Mechanical key for the generic identification of bacteria," our strains were confermed to belong to *Micrococcus*.

III. The characters of our strains

The characters of our 5 strains isolated independently and 19 identified species of micrococci were studied comparatively according to Hucker's system in Bergey's Manual. The characters of our glutamic acid accumulating micrococci are as follows.

I. Microscopic observation: Slightly ellipsoidal spheres, usually in pairs, and also in single and irregular masses. 0.6–1.2 μ. Ellongated forms can be observed under certain conditions. Not motile, spores are not formed, and Gram-positive.

II. Nutrient agar slant: Moderate, growth, filiform, dull, and milky white to pale yellow.

III. Nutrient agar colonies: Circular, smooth, perfect, slightly elevated, and milky white to pale yellow.

IV. Nutrient agar stab: Growth occurs only on the surface.

V. Nutrient broth: Slightly turbid, and rings are sometimes seen along tube. Flocculent sediment, and no odor.

VI. Physiological properties:

1) Temperature: No growth at 47°C, very slight growth at 42°C, and good growth at 28–37°C. Optimum temperature about 30°C.
2) pH: Growth in the range of pH 6–9 and optimum pH is 7–8.
3) Relation to free oxygen: Aerobic.
4) Nitrites produced from nitrates.
5) Hydrogen sulfide: Not formed.
6) Indole: Not formed.
7) Reduction of dyes: Methylene-blue, 2,6-dichlorophenolindophenol, Janus green, and 2,3,5-triphenyltetrazolium chloride are reduced.
8) Gelatin: Not liquefied or faintly liquefied.
9) Casein dissimilation: Negative or very weak.
10) Acetyl methyl carbinol: Not produced.
11) Methyl-red test: Weekly acidic.
12) Citrate utilization in Koser's medium: Negative.
13) Starch: Not liquefied.
14) Litmus milk: No change or slightly alkaline.
15) Catalase: Positive.
16) Urease: Strongly positive. (Christensen's[7] method and also by using cell suspension)
17) Phosphatase: Negative. (by the method of M. Barber et al.[8])
18) Mono-ammonium-phosphate is not utilized in Hucker's medium. When biotin is added, good growth is observed in Hucker's medium. Thus, it is clear that our strain requires biotin for its growth.
19) Production of organic acid: alpha-Ketoglutaric acid, and lactic acid are

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[4] C. E. Winslow, A. R. Winslow, "The systematic relationships of the coccaceae". John Wily & Sons. New York, (1908). (cited from references-3).

[5] Y. Abd-El-Malek and T. Gibson, *Jour. Dairy Research*, **15**, 249 (1948).

[6] V. B. D. Skerman, *Bact. Revs.*, **13**, 175 (1949).

[7] W. B. Christensen, *J. Bact.*, **52**, 461 (1952).

[8] M. Barber and S. W. A. Kuper, *J. Path. Bact.*, **63**, 65 (1951).

TABLE I. ACID PRODUCTION FROM CARBOHYDRATES BY M. 534

Carbohydrates	Agar media	Liquid media	Carbohydrates	Agar media	Liquid media
Inositol	—	±	Melezitose	—	—
Arabinose	—	±	Starch	—	—
Rhamnose	—	—	Inulin	—	—
Xylose	—	±	Dextrin	—	—
Glucose	+	+	Glycogen	—	—
Fructose	+	+	Glycerol	—	—
Galactose	—	—	Erythritol	—	—
Mannose	+	+	Adonitol	—	—
Lactose	—	—	Mannitol	—**	—**
Sucrose	+	+	Sorbitol	—	—
Maltose	+	+	Dulcital	—	—
Trehalose	—*	—	Salicin	—***	—
Melibiose	—	—	Aesculin	—	—
Cellobiose	—	—	α-Methylglucoside	—	—
Raffinose	—	—			

+ : Acid is produced.; — : Acid is not produced; ± : Acid production is doubtful *+ by M. 588, **+ by M 582, M. 516 and M. 566, ***+ by M. 560 and M. 588.

produced in glucose medium.

20) L-Glutamic acid is accumulated in a large quantity aerobically in the presence of carbohydrates, ammonium ion and inorganic salts.

21) Acid formation from carbohydrates: As shown in Table I, acid is produced from glucose, fructose, mannose, sucrose and maltose. Some strains produce acid from mannitol and salicin. In all cases gas formation is not recognized.

22) Habitat: May be air, soil and sewage.

IV. Decision of species

Our strains are aerobic, so *M. aerogenes*, *M. assacharolyticus*, *M. niger*, *M. grigoroffi*, and *M. anaerobius* were differentiated from our strains in their strict anaerobic character. *M. roseus*, *M. cinnabareus*, *M. rubens*, *M. rhodochrous* and *M. agilis* are different from our strains in their pink to red pigment formation on agar. Our strains are also different from *M. luteus*, *M. ureae*, *M. freudenreichii*, *M. flavus* and *M. candidus* in the respect that these species do not reduce nitrate to nitrite. Among those species which are aerobic and do not form red pigment on agar and reduce nitrates, *M. conglomeratus*, *M. varians*, and *M. caseolyticus* are different from our strains in their manners of ammonium salt utilization in Hucker's medium. *M. pyogenus var. aureus*, *M. pyogenes* var. *albus* and *M. citreus* are different from our strains in their gelatin liquefying character. So, only *M. aurantiacus* and *M. epidermidis* can remain as related species with our strains. But these species are found to be different from our strains in the following characters:

Media	*M. aurantiacus*	*M. epidermidis*	M. 534
Milk	Slightly acid.	Acid.	No change or slightly alkaline.
Lactose	Acid produced.	Acid produced.	No acid produced.
Nutrient broth	Turbid with pellicle.	Turbid with white ring and sediment.	Slightly turbid, rings are sometimes seen along the tube. Flocculent sediment.
Nutrient agar	Buff to scant orange-yellow beaded growth, raised, glistening.	White.	Moderate growth, milky white to pale yellow.

TABLE II—1. COMPARATIVE STUDIES ON PHYSIOLOGICAL CHARACTERISTICS OF OUR STRAINS AND OTHER *MICROCOCCI*

	Relation to free oxygen (+: aerobic)	Red pigment on ager	Nitrate reduction	Ammonium salt utilization	Yellow pigment	Milk**	Gelatin liquefaction	Indole formation	Hydrogen sulfide production	Starch hydrolysis	Urea utilization
M. luteus ATCC 398	+	−	−	−*	+	N	−		−		
M. freudenreichii ATCC 8459	+	−	−	−*	+	A	−		−		
M. caseolyticus ATCC 8460	+	−	+	−*	−	AC	+		−	−	
M. flavus	+	−	−	−	+	N	+		−	−	
M. conglomeratus	+	−	+	+	+	N	+		±	−	
M. varians ATCC 399	+	−	+	+	+	Al	+*		−		
M. varians Todai		−	−*	+	±	AC	−		−	−	+
M. pyogenes var. *aureus* 209 P		−	+	−	+	AC	+		−*	−	
M. pyogenes var. *albus*		−	+	−	−	A	+		−*	−	
M. pyogenes var. *albus* M-1-5		−	+	−	−	A	±		−*	−	
M. citreus		−	−*	−	±	N	+		−	−	−
M. citreus ATCC 4012		−	+	−	±	AC	−*		−	−	
M. citreus M-1-3		−	−*	−	+	N	+		−	−	−
M. percitreus		−		±	+	N	+		−	−	
M. epidermidis ATCC 155		−	+	±	−	A	−		−	±	
M. epidermidis Chiba Univ.		−	+	±	−	N	−		−	−	
M. lysodeikticus ATCC 4698		−	−	±	+	N	−		−	−	
M. lysodeikticus M-1-4		−	−	−	+	N	+		−	−	−
M. sodonensis	+	−	−	−	+	N	±		−	−	−
M. 534	+	−	+	−	±	N	−	−	−	−	−
M. 582	+	−	+	−	±	N	±	−	−	−	−
M. 516	+	−	+	−	±	N	−	−	−	−	−
M. 560	+	−	+	−	±	N	−	−	−	−	−
M. 588	+	−	+	−	±	N	−	−	−	−	−
M. roseus ATCC 9815		+	+	−*	−	N	−*				
M. rubens ATCC 186		+	+	−*	−	N*	−				
Sarcina lutea		−	−			Al					
Sarcina lutea S-1-6		−	−								

* Not conformed to Bergey's Manual 6th Ed.

** N: no change, A: acid, AC: acid clot Al: alkaline

Two strains of *M. epidermidis* which were tested were also differentiated from our strains in serological tests.

The results of our comparative tests between named species and our strains, i.e., M. 534, M. 582, M. 516, M. 560 and M. 588 are presented in Table II.

V. Cell elongation phenomenon of our strains.

The cells of our strains are slightly ellipsoidal as compared with common micrococci, and occasionally very elongated forms were observed. An elongated cell had a size of 0.7×3.5 (Fig. 1 and Fig. 2).

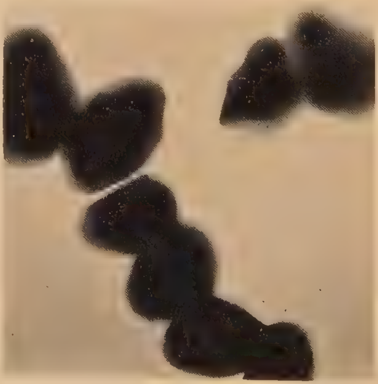

FIG. 1. Normal form (×12000)

TABLE II-2.

	Inositol	Arabinose	Rhamnose	Xylose	Glucose	Fructose	Galactose	Mannose	Lactose	Sucrose	Maltose	Trehalose	Melibiose	Gellobiose
M. luteus ATCC 398				±	±	−	−		−	−*	−			
M. freudenreichii ATCC 8459				−	+	+	+		+	+	+			
M. caseolyticus ATCC 8460	−	−	−	−	+	+	+	+	+	+	+	+	−	−
M. flavus	−	−	−	−	+	−	−	±	−*	+	+	−	−	−
M. conglomeratus	−	−	−	−	+	−	−	−	−*	−	−	−	−	−
M. varians ATCC 399				+	+	+	+		−*	+	−			
M. varians Todai	−	−	+	+	+	−	+	+	+	−	−	−	+	+
M. pyogenes var. *aureus* 209 P	−	−	−	−	+	+	+	+	+	+	+	+	−	−
M. pyogenes var. *albus*	−	−	−	−	+	+	+	+	+	−*	+	+	−	−
M. pyogenes var. *albus* M-1-5	−	−	−	+	+	+	+	+	+	+	+	+	+	+
M. citreus	−	−	−	−	−*	−	−	−	−*	−*	+	−	−	−
M. citreus ATCC 4012	−	−	−	−	+	+	+	+	+	+	+	+	−	−
M. citreus M-1-3	−	−	−	−	−*	−	−	−	−*	−*	−	−	−	−
M. percitreus	−	±	+	±	+	+	+	±	+	+	+	+	+	+
M. epidermidis ATCC 155	−	−	−	−	+	+	+	−	+	+	+	+	−	−
M. epidermidis Chiba Univ.	−	−	−	±	−*	−	−	−	−*	−*	±	+	−	−
M. lysodeikticus ATCC 4698	−	−	−	−	−	−	−	−	−	−	−	−	−	−
M. lysodeikticus M-1-4	−	−	−	−	−	−	−	−	−	−	−	−	−	−
M. sodonensis	−	−	−	−	+	−	−	+	−	+	+	±	−	−
M. 534	−	−	−	−	+	+	−	±	−	+	+	−	−	−
M. 582	−	−	−	−	+	+	−	+	−	+	+	−	−	−
M. 516	−	−	−	−	+	+	−	+	−	+	+	−	−	−
M. 560	−	−	−	−	+	+	−	+	−	+	+	−	−	−
M. 588	−	−	−	−	+	+	−	+	−	+	+	−	−	−
M. roseus ATCC 9815				+	+	+	−		−	±	±			
M. rubens ATCC 186				+	+	+	−		−	−*	−			
Sarcina lutea				+	+	+	−		−		−			
Sarcina lutea S-1-6				+	+	+	−		−		+			

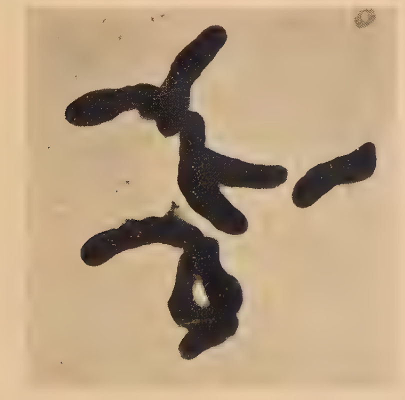

FIG. 2. Elongated form (×5000)

The reason of this elongation is yet not completely clarified. In some instances, however, the alkaline pH of the culturing medium seemed to be responsible for this phenomenon, but this did not always appear to be the case.

Cell elongation phenomenon in bacteria were recongnized in many species in relation to heavy metals, vitamins, antibiotics and other substances. Moreover, the general occurrence of pleomorphism of micrococci was reported by Shaw et al.[2] Taking these into consideration, the elongation phenomenon of our strains may not be the criteria to differentiate them from genus *Micrococcus*.

VI. The pigment of our strain M. 534

Colors of our strains show almost white to pale yellow on agar, and the pigmentations are very weak. In Hucker's classification system of micrococci in Bergey's Manual 6th. Ed., Pigmentation is an important character. In order to clarify the co-relation between

TABLE II-3

	Raffinose	Melezitose	Starch	Inulin	Dextrin	Glycogen	Glycerol	Erythritol	Adonitol	Mannitol	Sorbitol	Dulcitol	Salicin	Aesculin	α-Methylglucoside
M. luteus ATCC 398							−			−*	−		−		
M. freudenreichii ATCC 8459							+			+	−		+		
M. caseolyticus ATCC 8460	−	+	−	−	+	−	+	−	−	+	+	−	−	−	−
M. flavus	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
M. conglomeratus	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
M. varians ATCC 399							+			−	−		−		
M. varians Todai	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
M. pyogenes var. *aureus* 209 P	−	−	−	−	−	−	−*	−	−	+	−	−	−	−	−
M. pyogenes var. *albus*	−	+	−	−	−	−	−*	−	−	+	±	−	−	−	−
M. pyogenes var. *albus* M-1-5	+*	+	−	−	−	−	−	−	−	+	+	−	±	−	+
M. citreus	−	−	−	−*	−	−	−*	−	−	−*	−	−	−*	−	−
M. citreus ATCC 4012	−	±	−	−*	−	−	+	−	−	−*	−	−	−*	−	−
M. citreus M-1-3	−	−	−	−*	−	−	−*	−	−	−*	−	−	−*	−	−
M. percitreus	−	−	+	−	−	+	+	+	+	+	+	±	+	−	±
M. epidermidis ATCC 155	−	−	−	−	−	−	+	−	−	+*	−	−	−	−	−
M. epidermidis Chiba Univ.	−	−	−	−	−	−	−	+	±	±	−	−	−	−	−
M. lysodeikticus ATCC 4698	−	−	−	−	−	−	±	−	−	−	−	−	−	−	−
M. lysodeikticus M-1-4	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
M. sodonensis	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
M. 534	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
M. 582	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−
M. 516	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−
M. 560	−	−	−	−	−	−	−	−	−	+	−	−	+	−	−
M. 588	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−
M. roseus ATCC 9815							+		−*	+			−		
M. rubens ATCC 186							+		+	+			−		
Sarcina lutea							±		−	−			−		
Sarcina lutea S-1-6							+		−	−			−		

our strains and other micrococci and other genera in their pigmentation, characterization of pigment of our strains was carried out.

The cells grown on milk nutrient agar, and glucose agar, supplimented with yeast extract, were collected and washed lightly with acetone. After drying in vacuum, the pigments were extracted with methanol from the washed cells and absorption spectra of pigments were measured by a Beckman model DU spectrophotometer in methanol and chloroform solution. In this way, pigmented strains and deep yellow mutant U-2-17 (derived from M. 534 by UV irradiation) were tested.

As our strain M. 534 shows a weak pigmentation, a large quantity of cells grown in glucose nutrient medium by shaking culture were collected by centrifuge, then washed twice and extracted as mentioned above. The results of these experiments are shown in Table III and in Fig. 3. The pigment of our strain M. 534 showed no maximum at 415mμ but showed a shoulder at the same wave length. However, the pigment of U-2-17 showed a maximum at 415 mμ and seems to be the same xanthophyll of other yellow micrococci.

The relation between pigment and classification in bacteria is very important as it has been proposed in *Pseudomonas*,[9] *Neisseria*,[10]

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[9] W. C. Tobie, *J. Bact.*, **49**, 457 (1945).

[10] H. C. Ellinghausen, Jr., *J. Bact.*, **70**, 448 (1955)

TABLE II-4.

	Urease	Voges-Proskauer reaction	Methyl-red test	Phosphatase	Catalase	Agglutination with immune serum against M. 534	Precipitation with immune serum against M. 534
M. luteus ATCC 398	−	−	−	−	+	−	−
M. freudenreichii ATCC 8459	−	+	−	−		−	−
M. caseolyticus ATCC 8460	+	+	+	+		−	−
M. flavus	−	−	−	−	+	−	−
M. conglomeratus	−	−	−	−	+	−	−
M. varians ATCC 399	+	−	+	−	+	−	−
M. varians Todai	+	−	+	+	+	−	−
M. pyogenes var. *aureus* 209 P	±	+	+	+	+	−	−
M. pyogenes var. *albus*	+	±	±	−	+	−	−
M. pyogenes var. *albus* M-1-5	−	−	+	+	+	−	−
M. citreus	−	−	−	−	+	−	−
M. citreus ATCC 4012	+	+	+	+	+		
M. citreus M-1-3	+	−	−	−	+	−	−
M. percitreus	−	−	±	−			
M. epidermidis ATCC 155	+	+	+	−	+	−	−
M. epidermidis Chiba Univ.	−	+	±	−	+	−	−
M. lysodeikticus ATCC 4698	+	−	−	−	+	−	−
M. lysodeikticus M-1-4	+	−	−	−	+	−	−
M. sodonensis	−	−	−	−	+	−	−
M. 534	+	±	±	−	+	+	+
M. 582	+	+	+	−	+	+	+
M. 516	+	±	±	−	+	−	±
M. 560	+	±	±	−	+	−	±
M. 588	+	±	±	−	+	±	+
M. roseus ATCC 9815	−	−	−	±	+	−	−
M. rubens ATCC 186	−	−	±	±	+	−	−
Sarcina lutea	+	−	±	−	+		
Sarcina lutea S-1-6		−	±		+		

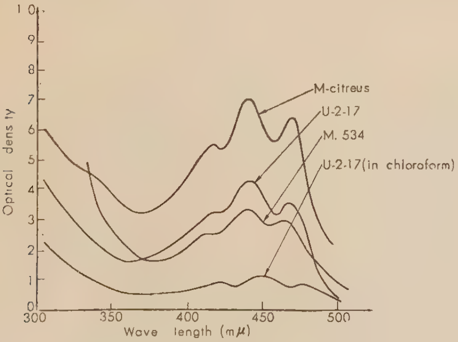

FIG. 3. Absorption spectra of pigments of several strains of Micrococcus in methanol solution.

and *Chromobacterium*.[11] On the yellow carotenoid of micrococci, Chargaff[12] obtained a hydrocarbon named sarcinene and a xanthophyll from *Sarcina lutea*. These pigments belong to group 2 and group 7 or 8 pigments in Sobin's[13] article, respectively. These groups are not differentiated by absorption maxima in alcohol solution. In our study the separation of xanthophyll from hydrocarbon was not attempted. But in light of the fact that Sobin et al, observed group 7 and 8 pigments in *M. luteus* and *M. flavus*, and Ellinghausen[10] measured the maxima of the pigment of *M. citreus* to be 410, 440 and 470mμ in methanol solution, the pigment of our

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[11] J. P. Gilman, *J. Bact.*, 65, 48 (1953).

[12] E. Chargaff and J. Dieryck, *Naturwissenschaften*, **20**, 872 (1932).

[13] B. Sobin, G. T. Stahley, *J. Bact.*, **44**, 265 (1942).

TABLE III. COMPARISON OF ABSORPTION MAXIMA OF THE PIGMENT OF M. 534 WITH THOSE OF OTHER MICROCOCCI

Strain	Absorption maxima in	
	Methanol mμ	Chloroform mμ
M. conglomeratus	414–15, 438–39, 468	
M. varians ATCC 399	〃 〃 〃	
M. lysodeikticus ATCC 4698	〃 〃 〃	
M. lysodeikticus M–1–4	〃 〃 〃	
M. luteus ATCC 398	〃 〃 〃	
M. sodonensis	〃 〃 〃	
M. flavus	〃 〃 〃	
M. citreus	〃 〃 〃	
M. citreus M–1–3	〃 〃 〃	
Sarcina lutea	〃 〃 〃	
Sarcina lutea M–1–6	〃 〃 〃	
M. 314, 307, 337, 2–3, 2–104, 303, 2–13, 2–41, 1–22, 231, 1–4, 310, and 354	〃 〃 〃	
M. 315	409–12, 438–39, 468	
M. 534	438–39, 468	
M. U–2–17	416–18, 438–40, 468	425, 445–48, ca. 475
M. roseus ATCC 9815	ca. 470, ca. 495	
M. rubens ATCC 186	ca. 469, ca. 493–94	

strains which showed absorption maxima at wave lengths of 414–15, 438, 468 mμ may be xanthophyll. Our observation and literature shows that most pigments of yellow micrococci are xanthophyll which had the same absorption maxima in alcohol solution.

The other genera in which the same pigments have been observed are *Flavobacterium*[14] and *Cellulomonas*.[13] According to R.S. Breed,[15] "rod-shaped organisms in *Achromobacteriaceae* possess many characteristics which are common to the spherical organisms belonging to genus *Micrococcus*. Habitat relationships even suggest their evolutionary development from salt-water form, one group having become adapted to life in soil and fresh water, and another to the life on the skin of fishes and then to life on the skin of higher vertabrates."

The fact that our strains are Gram-positive and *Flavobacterium* are generally Gram-negative, despite that our strains are ellipsoidal than usual micrococci, shows that our strains are micrococci related to *Flavobacterium* from Breed's opinion.

VII. Conclusion.

From the above experimental facts, our strains, M. 534, M. 582, M. 516, M. 560 and M. 588 belong to the same species. Since there are no species which conform to our strains in Bergey's Manual 5th Ed. and 6th Ed., our strains seem to be of a new species. Therefore we propose to give the name, *Micrococcus glutamicus* nov. sp. to our strains, in relation to their marked character of glutamic acid accumulation.

SUMMARY

1) Newly isolated glutamic acid accumula-

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[14] D. P. Couringtan and T. W. Goodwin, *J. Bact.*, **70**, 568 (1955).

[15] R. S. Breed, *J. Bact.*, **50**, 125 (1954).

ting bacteria involving 5 strains were studied according to the system described in Bergey's Manual 6th Ed., and named *Micrococcus glutamicus* nov. sp..

2) The cell elongation phenomenon and absorption spectrum of the pigment of *M. glutamicus* nov. sp. was studied in relation to its classification. The cell elongation especially, occurred in urea medium. The pigment of *M. glutamicus* seems to belong to xanthophyll as those of other yellow micrococci.

Acknowledgment The authors are grateful to Dr. B. Kato, President of our company, Mr. H. Watanabe and Mr. N. Sato. We also wish to thank Dr. S. Abe and Members of our laboratory for their kind advice.

[Bull. Agr. Chem. Soc. Japan, Vol. 22, No. 3, p. 185~193, 1958]

Relationship between Stereoisomerism and Biological Activity of Pyrethroids.

Part I. Synthesis of Homochrysanthemic Acid and Related Compounds.

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Received December 29, 1957

(±)-*trans*-Homochrysanthemic acid, m.p. 80–80.5°, is obtained by the Arndt-Eistert reaction of (±)-*trans*-chrysanthemoyl chloride with diazomethane and the structure of the resulting homo-acid is confirmed by ozonolysis to give acetone and (±)-*trans*-homocaronic acid. (±)-*cis*-Chrysanthemoyl chloride, when undergoing the same reaction sequence, gives (±)-*cis*-3-*iso*butenyl-2,2-dimethyl*cyclo*butane-1-carboxylic acid, bp. 104–5°/2 mm., which is demonstrated by ozonolysis to give acetone and *cis*-norpinic acid. Both (±)-*trans*-homochrysanthemic and (±)-*cis*-3-*iso*butenyl-2,2-dimethyl-*cyclo*butane-1-carboxylic acids are readily converted by boiling with dilute sulphuric acid into the same δ-lactone. This lactone is theoretically deduced to be δ,δ-dimethyl-γ-*iso*butenyl-δ-valerolactone and is also confirmed by ozonolysis to give acetone and δ,δ-dimethyl-γ-carboxy-δ-valerolactone, m.p. 175°.

Relationship between chemical structure and biological activity of the "Pyrethrins" has been discussed by many workers on the naturally derived "pyrethrins" and many synthetic pyrethroids in which various molecular modifications are introduced. Some features in both acid and alcohol moieties of the pyrethroids have been concluded to be indispensable for the high activity. During the last decade a remarkable advance in this field of pyrethrin chemistry has added further information, thus making a number of synthetic stereoisomeric pyrethroids available for biological test. According to the bioassays by Gersdorff and Milton[1], with isomeric forms of allethrin, for example, (+)-allethronyl-(+)-*trans*-chrysanthemate, of the same optical series as the naturally occurring "pyrethrins," is deduced as five hundred times toxic as (−)-allethronyl-(−)-*cis*-chrysanthemate. This evidently indicates that the biological action of pyrethroids is closely associated with the particular stereochemical conformation in which the various parts, indispensable for toxicity, of the molecule are held in respect to each other and with the chemical and physical properties of the groups so positioned. Such a conception of the molecular conformation has been suggested by Ohono[2] and others.

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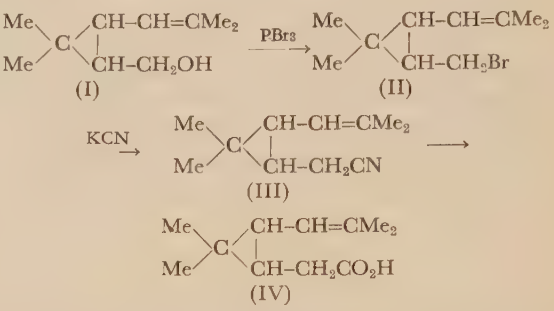
* Instituter of Chemical Research, Kyoto University.

[1] W. A. Gersdorff and N. Milton, *J. Was. Acad. Sci.*, **42**, 313 (1952).

One explanation for the specific difference in activity between stereoisomeric pyrethroids is that only in the highly active isomer, the indispensable groups could be all accomodated closely on the biological surface for activity to be manifested, whilst in the poor performing isomer, neither of the groups could closely fit on to appropriate positions the active centres in the biological system, or only a few groups could be in contact with the site of action, keeping the other groups completely out of contact. In the molecule of pyrethroids, free rotation can occur at several bonds and especially those connecting *cyclo*propane carboxyl group to *cyclo*pentenolone can affect to a great extent the molecular conformation. Thus, lengthening the ester linkage by one methylene group seems to be of interest from this point of view and this might improve the poor performance of (−)-*cis*-acid-(−)-alcohol combination, thereby providing experimental evidence for the suggested theory of molecular conformation. For this purpose, synthesis of higher homologues of chrysanthemic acids was undertaken.

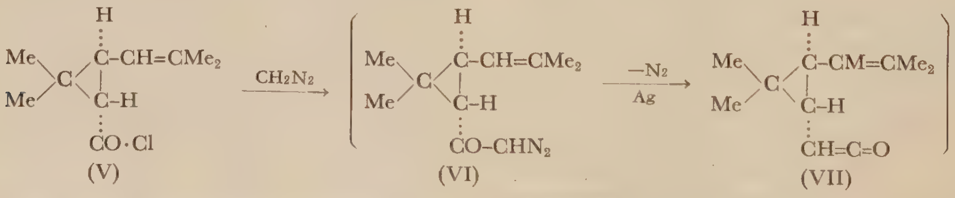
At first, the following scheme of synthesis was investigated.



trans-Chrysanthemol[3] was converted into the bromide thence to the nitrile, followed by acid hydrolysis to afford the homo-acid. However, the nitrile was difficulty hydrolyzable, and by unexplainable reasons the majority was reconverted into the parent *trans*-chrysanthemic acid, yielding a product consisting of chrysanthemic acid in main and the desired homo-acid in a small proportion. The yield of hydrolysis was not significantly improved by varying the conditions so this route did not seem to offer promise for the preparation of considerable quantities of the homo-acid required for further investigations.

At this time, the authors' attention was directed to the Arndt-Eistert reaction as an alternative route to the desired acids. It is with this work that the present paper is chiefly concerned.

The Arndt-Eistert reaction of (±)-*trans*-chrysanthemoyl chloride (V) with diazomethane gave, as was expected, (±)-*trans*-homochrysanthemic acid (VIII), m. p. 80–80.5°, in a superior yield.



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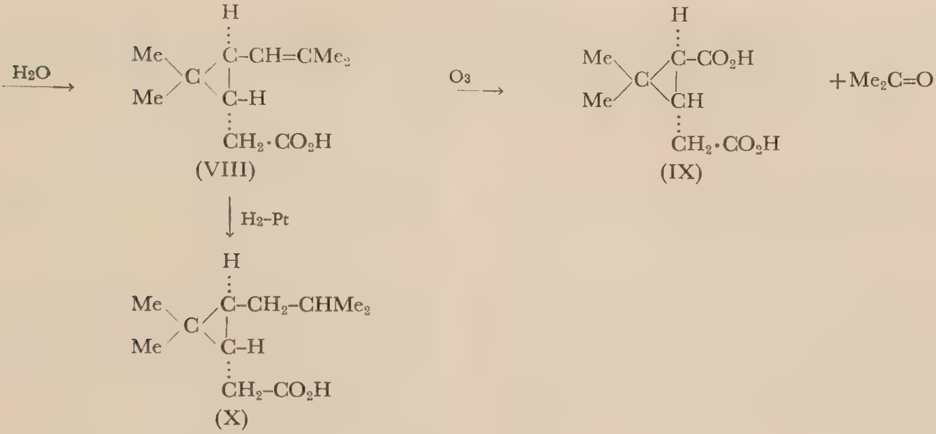
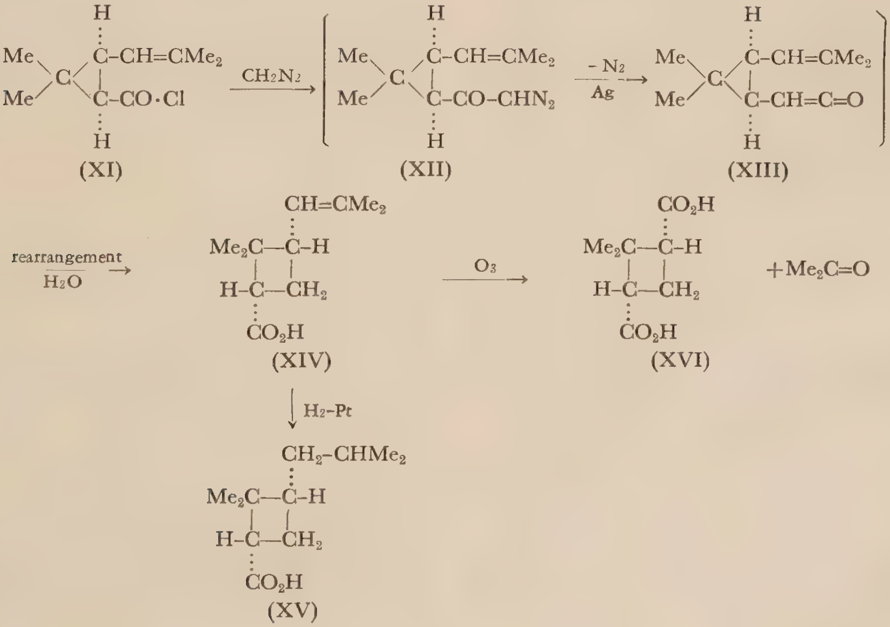
[2] M. Ohono, Lecture read before the 55th Semi-annual Meeting of Inst. Chem. Res., Kyoto Univ. (June, 11, 1955).

[3] Y. Inouye and M. Ohono, This Bulletin, 20, 25 (1956)



This acid absorbed bromine instantly and decolourlized permanganate solution. On catalytic hydrogenation over Adams' platinum catalyst. it took up 1 mole. of hydrogene, giving (±)-*trans*-dihydrohomochrysanthemic acid (X). The structure was confirmed by ozonolysis to give acetone (as 2,4-dinitrophenylhydrazone, m.p. 126°) and (±)-*trans*-homocaronic acid (IX), m.p. 190–191°. (±)-*trans*-Homochrysanthemic acid was also characterized by its *p*-bromophenacylester, m.p. 38–9°, and anilide, m.p. 112.5–114°.

In the case of *cis*-isomer, however, a different situation arose. (±)-*cis*-Chrysanthemoyl chloride (XI), upon undergoing the same reaction sequence, gave (±)-*cis*-3-*iso*butenyl-2,2-dimethyl*cyclo*butane-1-carboxylic acid exclusively (XIV), m.p. 104–5°/2 mm.



This liquid acid absorbed bromine and decolourized permanganate solution. Catalytic hydrogenation over Adams' platinum catalyst gave dihydroacid (XV), 1 mole. of hydrogen being taken up.

Ozonolysis XIV gave acetone (as 2,4-dinitrophenylhydrazone) and *cis*-norpinic acid (XVI),4) m.p. 173–5°, and not the expected (±)-*cis*-homocaronic acid thereby confirming the structure as XIV. This acid was also characterized by its *p*-bromophenacylester m. p. 43–4° and anilide, m.p. 84–6°. *cis*-Norpinic acid (XVI) was characterized by monoanilide, m.p. 212–3° which was consistent with that in the literature.5)

Such an anomaly of the Arndt-Eistert reaction as encountered in the present case where the reaction proceeds towards ring expansion and not towards normal extention of carbon chain, has so far not, been reported. This is evidently ascribed to the steric effect that the proximity of the bulky *iso*butenly group to the carboxyl positioned in *cis* with respect to each other, prevents the normal addition in Arndt-Eistert reaction, since this is not the case with the *trans*-acid which gives the normal homo-acid by the same procedures. This anomaly surmises some similarity in mechanism to the ring expansion of carbocyclic ketones by the action of diazomethane.6) In the present case, the *cis*-configuration is retained as such throughout the reaction and this might probably suggest that the molecular rearrangement towards ring expansion takes plase at the last stage, XIII →XIV, with simultaneous addition.

Matsui7) has attempted the Arndt-Eistert synthesis of *cis*-homochrysanthemic acid and obtained a liquid acid, b.p. 145–6°/14 mm., from which a crystalline acid, m.p. 78–9° (*p*-bromophenacylester m.p. 47–55°) was separated in a poor yield. Although he did not prosecute further study on structural problems of his acids and direct comparison with the present authors' products is lacking his crystalline acid may probably be identical with (±)-*trans*-homochrysanthemic acid (VIII) and not (±)-*cis*-homochrysanthemic acid as it was claimed by him. It seems likely that his crystalline acid may be derived from *trans*-chrysanthemic acid contaminated in the starting *cis*-chrysanthemic acid which he employed. The liquid acid fraction consituting the main product of his synthesis is assumed to be *cis*-3-*iso*butenyl-2, 2-dimethyl*cyclo*butane-1-carboxylic acid (XIV).

The ease with which (±)-*trans*-homochrysanthemic acid (VIII) and (±)-*cis*-3-*iso*butenyl-2, 2-dimethyl*cyclo*butane-1-carboxylic acid (XIV) are lactonized in boiling dilute sulphuric acid seems of particular interest and worthy of note.

In boiling 5% sulphuric acid VIII was readily lactonized to the liquid lactone (XVII), b.p. 88°/2 mm., in contrast to (±)-*trans*-chrysanthemic acid which is converted under similar conditions into the hydrated acid, (±)-*trans*-δ-hydroxydihydrochrysanthemic acid. Examination of the molecular model of VIII indicates that the carboxyl and *iso*butenyl groups are so far apart to form ε-lactone much less probable if not impossible (highly strained structure should be postulated) and then, it necessitates that the formation of the lactone from VIII may be due to the fission of *cyclo*propane ring through simultaneous intramolecular interaction of the carboxyl group. Consistent with this prediction, the infra-red absorption spectrum of the lactone showed the characteristic bands for tri-substituted ethylene ($835\,\text{cm}^{-1}$) and δ-lactone ($1755\,\text{cm}^{-1}$). In fact, the lactone absorbed bromine but slowly, and on hydrogenation over Adams' platinum catalyst, it gave the dihydrolactone, just 1 mole. of hydrogen being taken up. Ozonolysis of the lactone gave acetone and crystalline δ-lactonic acid (XIX), m. p. 175°. Fission of the δ-lactone ring of XIX to give a hydroxy-acid, (XX) was effected by alka-

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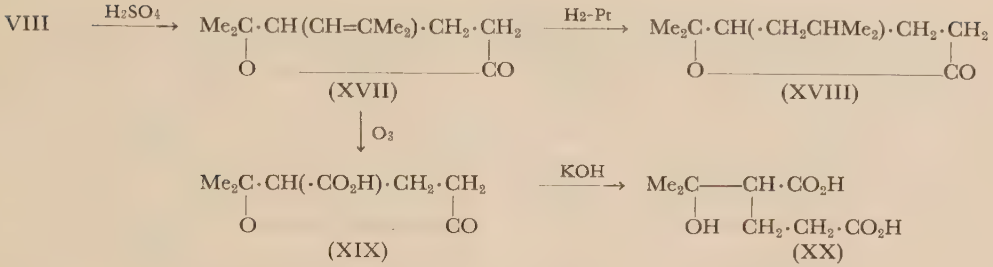
4) P. G. Guha and K. Ganapathi, *Ber.*, **69**, 1185 (1936).

5) M. Kerschbaum, *Ber.*, **33**, 891 (1900).

6) C. D. Gutsche, *Organic Reactions*, Vol. VIII, 377 (1954).

7) M. Matsui and S. Kitamura, This Bulletin, **19**, 42 (1955).



$$\mathrm{VIII}\xrightarrow{H_2SO_4}\underset{(\mathrm{XVII})}{\begin{array}{l}\mathrm{Me_2C\cdot CH(CH{=}CMe_2)\cdot CH_2\cdot CH_2}\\ \mathrm{O}\text{————————}\mathrm{CO}\end{array}}\xrightarrow{H_2\text{-}Pt}\underset{(\mathrm{XVIII})}{\begin{array}{l}\mathrm{Me_2C\cdot CH(\cdot CH_2CHMe_2)\cdot CH_2\cdot CH_2}\\ \mathrm{O}\text{————————}\mathrm{CO}\end{array}}$$

$$\downarrow O_3$$

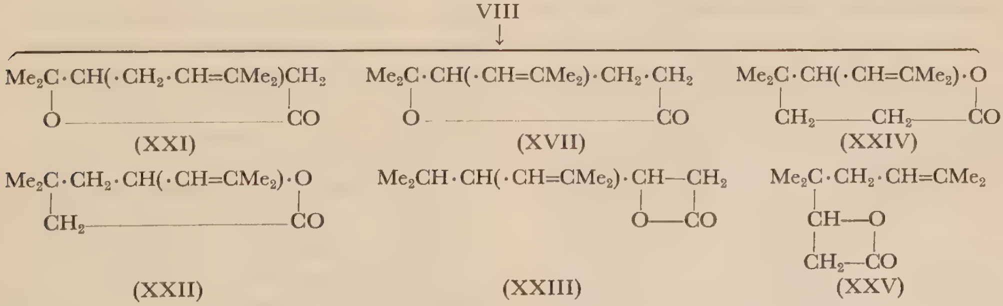
$$\underset{(\mathrm{XIX})}{\begin{array}{l}\mathrm{Me_2C\cdot CH(\cdot CO_2H)\cdot CH_2\cdot CH_2}\\ \mathrm{O}\text{——————}\mathrm{CO}\end{array}}\xrightarrow{KOH}\underset{(\mathrm{XX})}{\begin{array}{l}\mathrm{Me_2C\text{——}CH\cdot CO_2H}\\ \mathrm{OH\quad CH_2\cdot CH_2\cdot CO_2H}\end{array}}$$

line hydrolysis and the resulting hydroxy-acid, though not isolated pure, gave, with Denige's reagent, a positive test for a tertiary hydroxyl group and negative with Nessler's reagent.

Of the six structures* possible for the lactone to be derived from VIII by the *cyclo*-propane-ring fission through intra molecular attack of carboxyl group, XXII and XVII can suffice the experimental requirements mentioned above. Structure XXII, on ozonolysis, leads to terpenylic acid and is reasonably excluded by the melting point discrepancy with the existing lactonic acid. By exclusion, therefore, the lactonic acid melting at 175° is concluded to be δ,δ-dimethyl-γ-carboxy-δ-valerolactone and hence, the lactone to be δ,δ-dimethyl-γ-*iso*butenyl-δ-valerolactone. This is a homologue of (±)-pyrocin whose structure was shown by Crombie[8] to be β-*iso*butenyl-*iso*hexano-γ-lactone and behaves similarly to pyrocin.

(XIV) gave, in boiling dilute sulphuric acid, the same lactone (XVII) as was derived from VIII. The yield of XVII from XIV was also superior. Identity of the lactone from the two different sources was provided by the complete identity of infra-red spectra, boiling points, refractive indices and also by ozonolysis to give acetone and the lactonic acid (XIX). The before-mentioned deduction of structure XVII for the lactone is furthermore justified by formation of the same lactone from this differently structured acid (XIV) amongst the six structures theoretically possible for the lactone to be derived from XIV, only the structure XVII does exist in commom with one among those from VIII.

Owen and Simonsen[9] obtained a lactonic acid $C_8H_{12}O$, m.p. 101–2°, by heating (±)-*cis*-homocaronic acid with hydrochloric acid at 100°. The constitution of the lactonic acid was not determined by them but was considered to be either XIX or XXXI.



$$\mathrm{VIII}\downarrow$$

$$\underset{(\mathrm{XXI})}{\begin{array}{l}\mathrm{Me_2C\cdot CH(\cdot CH_2\cdot CH{=}CMe_2)CH_2}\\ \mathrm{O}\text{————————}\mathrm{CO}\end{array}}\qquad\underset{(\mathrm{XVII})}{\begin{array}{l}\mathrm{Me_2C\cdot CH(\cdot CH{=}CMe_2)\cdot CH_2\cdot CH_2}\\ \mathrm{O}\text{————————}\mathrm{CO}\end{array}}\qquad\underset{(\mathrm{XXIV})}{\begin{array}{l}\mathrm{Me_2C\cdot CH(\cdot CH{=}CMe_2)\cdot O}\\ \mathrm{CH_2\text{——}CH_2\text{——}CO}\end{array}}$$

$$\underset{(\mathrm{XXII})}{\begin{array}{l}\mathrm{Me_2C\cdot CH_2\cdot CH(\cdot CH{=}CMe_2)\cdot O}\\ \mathrm{CH_2}\text{————————}\mathrm{CO}\end{array}}\qquad\underset{(\mathrm{XXIII})}{\begin{array}{r}\mathrm{Me_2CH\cdot CH(\cdot CH{=}CMe_2)\cdot CH\text{—}CH_2}\\ \mathrm{O\text{—}CO}\end{array}}\qquad\underset{(\mathrm{XXV})}{\begin{array}{l}\mathrm{Me_2C\cdot CH_2\cdot CH{=}CMe_2}\\ \mathrm{CH\text{—}O}\\ \mathrm{CH_2\text{—}CO}\end{array}}$$

Surprising is the fact that (±)-*cis*-3-*iso*butenyl-2,2-dimethyl-*cyclo*butane-1-carboxylic acid

However an additional structure XXXII is theoretically possible, terpenylic acid and unlikely β-lactonic acid being excluded.

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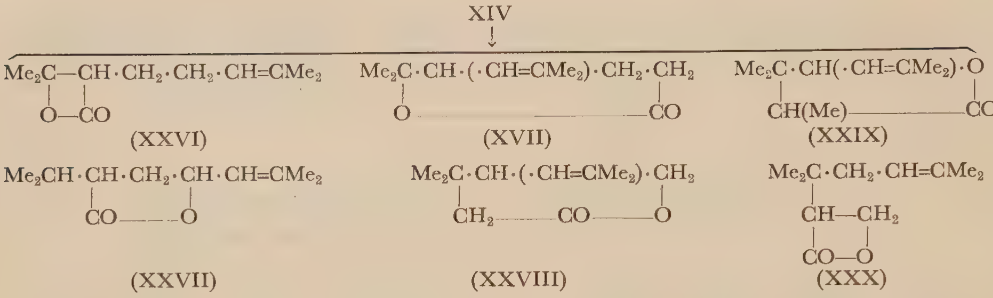
* Improbable formation of β-lactone being excluded.

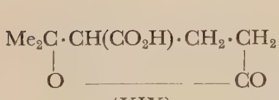
[8] L. Crombie, S. H. Harper and R. A. Thompson, *J. Sci. Food Agr.*, **1951**, 421.

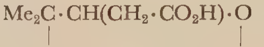
[9] J. Owen and J. L. Simonsen, *J. Chem. Soc.*, **1933**, 1225.

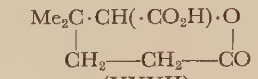

XIV

(XXVI) (XVII) (XXIX)

(XXVII) (XXVIII) (XXX)


(XIX)


(XXXI)


(XXXII)

Of the three, XIX, now designated for the lactonic acid, m.p. 175°, and XXXI, m.p. 154–6°, prepared by Perkin jun.[10] are excluded from melting points discrepancies. By exclusion, therefore, Simonsen's lactonic acid must be XXXII, i. e. γ,γ-dimethyl-δ-carboxy-δ-valerolactone.

It is noticable that both (±)-*trans*-homochrysanthemic and (±)-*cis*-3-*iso*butenyl-2, 2-dimethyl*cyclo*butane-1-carboxylic acids are readily lactonized into a *tert.*-δ-lactone with *cyclo*propane and *cyclo*butane ring opened under the milder hydrolytic conditions, in contrast to much more drastic conditions required for the lactonization of chrysanthemic acid into (±)-pyrocin and of caronic acid to terebic acid. At any rate, the lactonization in all these cases did ~~not~~ occur with the ring fission at the bond connecting the gem.-dimethyl carbon $C_{(2)}$ to that substituted by carboxyl or carboxymethyl $C_{(1)}$, through intramolecular attack of the carboxyl group, except in the case of homocaronic acid where $C_{(1)}$–$C_{(3)}$ bond is broken to give *sec*-lactonic acid.

Alternative route of synthesis for *cis*-homochrysanthemic acid is actively in hand. Bioassays of the optically isomeric esters of *trans*-homochrysanthemic acids with allethrolones are now in progress and will be published elsewhere in the near future.

EXPERIMENTAL

Melting and boiling points were uncorrected. Microanalyses were carried out by the Microanalytical Division of Mitsui Laboratory of Kyoto University, to whom the authors' thanks are due. Infra-red spectra were determined with a Shimadzu double beam spectrophotometer (Model AR–275).

(±)-*trans*-**Chrysanthemyl Bromide** (II) (±)-*trans*-Chrysanthemol (23 g), prepared by the procedure previously described,[3] mixed with dry pyridine (4 ml.) in benzene (7 ml.), was cooled in icesalt mixture, and to this, phosphorous tribromide (16 g., 1.2 mole.) in benzene (10 ml.) was added dropwise at −20° with stirring for one hour and left standing overnight at room temperature. The bromide was distilled over direct flame and boiled mainly at 55–75°/10 mm. (30 g.). After being washed twice with cold 10% sodium hydroxide and twice with 5% sulphuric acid, light petroleum (b.p. 40–60°) being used to aid separation, the product was dried and distilled to yield (±)-*trans*-chrysanthemyl bromide, b. p. 88–9°/23mm., n_D^{20} 1.4758 (22 g., 68%).

(±)-*trans*-**Chrysanthemyl Nitrile** (III) In a three-necked flask, were placed potassium cyanide (27 g.), water (4 ml.) and ethanol (10 ml.). The flask was heated in a boiling water bath and the contents were stirred until all the sodium cyanide was dissolved. Then (±)-*trans*-chrysanthemyl bromide (8g.) was added slowly, and stirring and heating were continued for three hours. After ethanol was removed, the residue was extracted with benzene and the benzene solution was washed well with water and dried over calcium chloride. After removal of the solvent, the residue was distilled under reduced pressure to yield (±)-*trans*-chrysanthemyl nitrile, b. p. 62–4°/10 mm., (5.0 g. 84 %).

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[10] W. H. Perkin jun. and J. F. Thorpe, *J. Chem. Soc.*, **1913**, 1760.

Hydrolysis of (±)-*trans*-Chrysanthemyl Nitrile. The nitrile (4g.) was boiling with 25ml. of 2N-ethanolic potassium hydroxide for 15 hours. After the evolution of ammonia ceased, the mixture was concentrated *in vacuo* and the hydrolyzate was extracted with ether to remove the neutral substance and then acidified.

Extraction with ether and evaporation of the solvent gave a crystalline mass (3g.). On separative crystallization from light petroleum, a small amount of homo-acid, m.p. 80–80.5° (sec. below) and the parent (±)-*trans*-chrysanthemic acid (ca. 2 g.) were obtained. Acid hydrolysis of then nitrile did not improve the yield of the desired homo-acid.

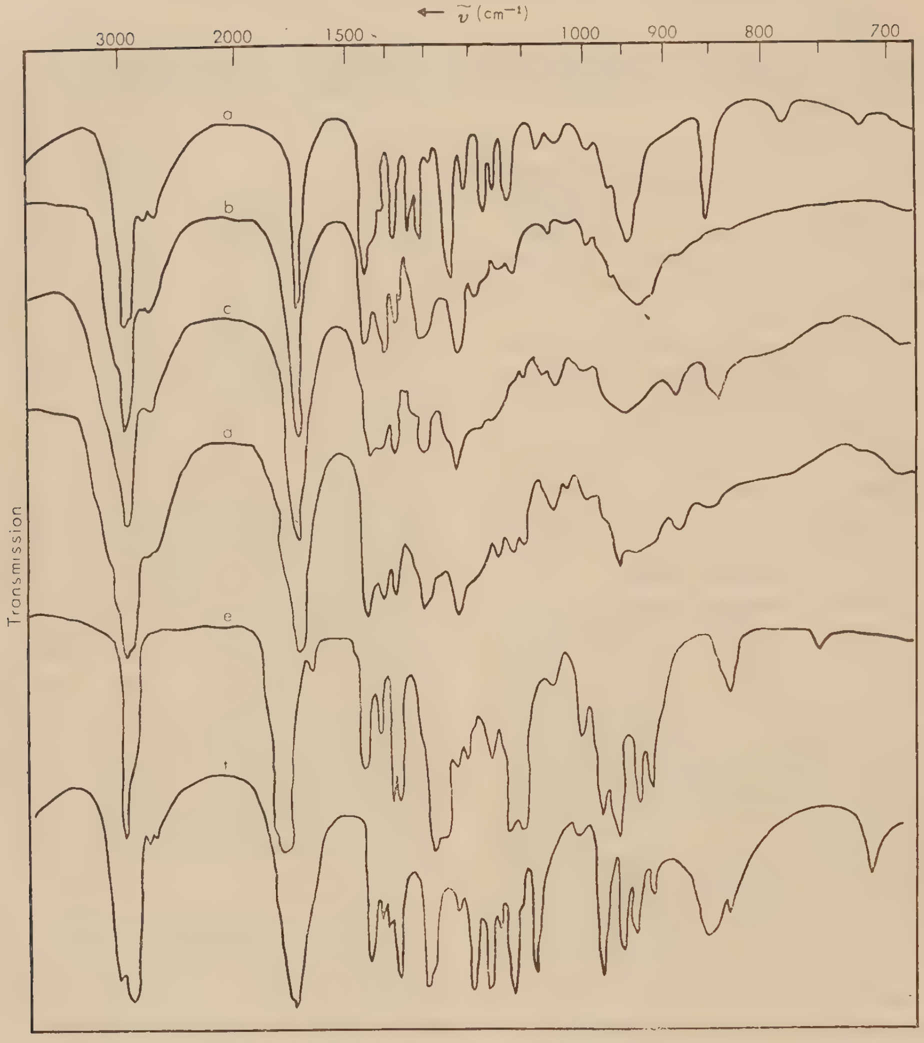
(±)-*trans*-Homochrysanthemic Acid (VIII) A solution of 18 g. of (±)-*trans*-chrysanthemoyl chloride in 30 ml. of absolute ether was added at 0–5° to a solution of diazomethane prepared from 50g. of nitrosomethylurea in 500 ml. of ether. After three hours at 20–25°, the ether was removed under reduced pressure finally at 30°. The yellow residue of (±)-*trans*-chrysanthemoyl diazomethane (24g.) was obtained. A solution of the crude diazoketone in 100 ml. of dioxane was treated with 175 ml. of aqueous ammonia (sp. gr. 0.898) and 37 ml. of 10% aqueous silver nitrate solution at 60–70°. The mixture was boiled under reflux for three hours and the dioxane was distilled off. The *trans*-homochrysanthemic amide was extracted with ether and the ether was removed to yield the crude amide (23 g.). The amide was hydrolyzed with 10% methanolic sodium hydroxide for 12 hours. Then, 100 ml. of water were added and the solvent was distilled off. The mixture was extracted with ether, and the neutral substances were discarded. The alkaline solution was acidified and extracted with ether. Washing and distillation of the organic layer gave (±)-*trans*-homochrysanthemic acid, b. p. 106–7°/2 mm., m. p. 80–80.5°, equivalent weight by titration, Found 182.5 Calcd. for $C_{10}H_{17}CO_2H$. 182.3, (*Anal.* Found : C, 72.35 ; H, 9.77 ; Calcd. for $C_{11}H_{18}O_2$: C, 72.49 ; H, 9.96) Yield 14.5 g., (82 % based on acid chloride). This acid absorbed bromine instantly and decolourlized permanganate solution. For infra-red spectrum, see Fig. in the text. By the standard method, were prepared anilide, m.p. 112.5–114° (*Anal.* Found : C, 79.30 ; H, 9.27 ; N, 5.51 ; Calcd. for $C_{17}H_{23}ON$: C, 79.33 ; H, 9.01 ; N, 5.44) and *p*-bromophenacylester, m.p. 38–9° (*Anal.* Found : C, 60.43 ; H, 6.07 ; Calcd. for $C_{19}H_{23}O_3Br$: C, 60.16 ; H, 6.07)

Hydrogenation (±)-*trans*-Homochrysanthemic acid (0.9 g.) in 20 ml. of ethyl acetate was hydrogenated over Adams' platinum oxide catalyst in a shaking hydrogenation apparatus and absorbed 119ml. (at 16.5°) of hydrogen (equivalent to 1 mole), giving (±)-*trans*-dihydro-homochrysanthemic acid, b.p. 99°/2 mm., n_D^{20} 1.4461, equivalent weight by titration, Found 184.1 Calcd. for $C_{10}H_{19}CO_2H$. 184.3. For infra-red spectrum, see Fig. in the text. *p*-Bromophenacylester, prepared by the standard method, formed fine needles from ethanol, m.p. 51° (*Anal.* Found : C, 59.98 ; H, 9.63 ; Calcd. for $C_{19}H_{25}O_3Br$: C, 59.84 ; H, 6.61).

Ozonization (±)-*trans*-Homochrysanthemic acid (1.0 g.) dissolved in chloroform (20 ml.) was ozonized at 0°. The solvent was evaporated *in vacuo* and the residual ozonide was decomposed with water (40 ml.) and left standing overnight. Then the solution was distilled, three successive 5 ml. portions of distillate being collected. Treatment with an excess of 2, 4-dinitrophenylhydrazine in 2 N-hydrochloric acid gave acetone-2,4-dinitrophenylhydrazone. m. p. 126° ; not depressed by the admixture with the authentic specimen. Concentration of the remaining undistilled solution to 10 ml., gave *trans*-homocaronic acid (0.6 g.), m.p. 190–91°, equivalent weight by titration, Found 86.0 Calcd. for C_6H_{10} $(CO_2H)_2$. 86.1, (*Anal.* Found : C, 55.60 ; H, 7.03 ; Calcd. for $C_8H_{12}O_4$: C, 55,80 ; H, 7.03).

(±)-*cis*-3-*iso*Butenyl-2, 2-dimethyl-*cyclo*butane-1-carboxylic Acid By the same procedures as in (VIII), (±)-*cis*-chrysanthemoyl chloride (28.0 g.) and diazomethane (prepared from 78 g. of nitrosomethylurea) gave (±)-*cis*-3-*iso*butenyl-2,2-dimethyl*cyclo*butane-1-carboxylic acid, b.p. 104–5°/2 mm., n_D^{20} 1.4780, (14 g., 51 %). The equivalent weight by titration was 183.1 (Calcd. for $C_{10}H_{17}CO_2H$. 182.3). For infra-red, see Fig. in the text. By the standard method, anilide, m.p. 84–6° (*Anal.* Found : C, 79.30 ; H, 8.89 ; N, 5.50 ; Calcd. for $C_{17}H_{23}ON$: C, 79.33 ; H, 9.01 ; N, 5.44) and *p*-bromophenacylester, m.p. 43–4° (*Anal.* Found : C, 60.70 ; H, 6.13 ; Calcd. for $C_{19}H_{23}O_3Br$: C, 60.16 ; H, 6.07) were prepared.

Hydrogenation (±)-*cis*-3-*iso*Butenyl-2,2-dimethyl-*cyclo*butane-1-carboxylic acid (0.9 g.) in 20 ml. of ethy acetate was hydrogenated over Adams' platinum oxide catalyst and 115 ml. (at 16.5°) of hydrogen (equivalent to 1 mole.) were taken up giving a yield of the dihydro-



Infra-red spectra of a) (±)-*trans*-Homochrysanthemic acid (Nujol) b) (±)-*trans*-Dihydrohomochrysanthemic acid (liquid) c) (±)-*cis*-3-*iso*Butenyl-2, 2-dimethyl*cyclo*butane-1-carboxylic acid (liquid) d) (±)-*cis*-3-*iso*Butyl-2, 2-dimethyl*cyclo*butane-1-carboxylic acid (liquid) e) (±)-γ-*iso*Butenyl-δ, δ-dimethyl-δ-valerolactone (liquid) f) (±)-γ-Carboxy-δ, δ-dimethyl-δ-valerolactone (Nujol)

acid, b.p. 97–8°/2 mm. n_D^{20} 1.4532; equivalent weight by titration Found. 184.0 Calcd. for $C_{10}H_{19}CO_2H$. 184.3. For infra-red spectrum, see Fig. in the text.

Ozonization (±)-*cis*-3-*iso*Butenyl-2,2-dimethyl-*cyclo*butane-1-carboxylic acid (1.0 g.) dissolved in chloroform (20 ml.) was treated with an excess of ozon at 0°. The solvent was evaporated under reduced pressure and the residual ozonide was decomposed with water (40 ml.). From the distillate, acetone was isolated as 2,4-dinitrophenylhydrazone, m.p. 126° (mixed). On concentration of the remaining solution to 10ml., *cis*-norpinic acid crystallized in prisms (0.85 g.), m.p. 173–5°, (*Anal.* Found: C, 55.71; H, 7.16; Calcd.

for $C_8H_{12}O_4$: C, 55.80; H, 7.03), equivalent weight by titration, Found 86.0 for $C_6H_{10}(CO_2H)_2$ 86.1. monoanilide, m.p. 212–3° (*Anal.* Found: C, 67.79; H, 7.01; N, 5.71; Calcd. for $C_{14}H_{17}O_3N$: C, 68.00; H, 6.93; N, 5.66). This acid is completely consistent in every respect with that in the literature.[4]

δ,δ-Dimethyl-γ-*iso*butenyl-δ-Valerolactone (XVII) **a)** From (±)-*trans*-homochrysanthemic acid. (±)-*trans*-Homochrysanthemic acid (3.0 g.) was refluxed with (5%) (v/v) sulphuric acid (65 ml.) for three hours. The reaction mixture was taken up in ether, washed three times with 5% sodium hydroxide, then with water, and the ether solution was dried over anhydrous sodium sulphate. Distillation then gave δ,δ-dimethyl-γ-*iso*butenyl-δ-valerolactone, b.p. 88°/2mm., n_D^{20} 1.4599; for infra-red spectrum, see Fig. in the text. yield 2.5g. The lactone on boiling for 2 minutes with Deninge's reagent gave a brown precipitate and absorbed bromine slowly.

b) From (±)-*cis*-3-*iso*butenyl-2,2-dimethyl*cyclo*butane-1-carboxylic acid. By the same procedure **a)**, (±)-*cis*-3-*iso*butenyl-2,2-dimethyl*cyclo*butane-1-carboxylic acid (3.0 g.) gave (±)-δ,δ-dimethyl-γ-*iso*butenyl-δ-valerolactone. b.p. 88°/2 mm., n_D^{20} 1.4595 yield 2.5g. This was identical with the lactone described above, that was shown by the identies of boiling point, refractive index and infra-red spectrum, as well as the ozonolysis products (see below).

Hydrogenation (±)-δ, δ-Dimethyl-γ-*iso*butenyl-δ-valerolactone (0.5 g.) in 10 ml. of ethyl acetate was hydrogenated over Adams' platinum oxide catalyst and absorbed 1 mole. equivalent of hydrogen (68 ml. at 16.5°), yielding the dihydrolactone, b.p. 88–9°/2mm., n_D^{20} 1.4487.

Hydrolysis δ, δ-Dimethyl-γ-*iso*butenyl-δ-Valerolactone was hydrolyzed with 0.35 N-ethanolic potassium hydroxide for an hour, and was treated in the usual manner, but the cerresponding hydroxy-acid did not crystallize thus being, was converted into *p*-bromophenacylester m.p. 143° (*Anal.* Found: C, 57.61; H, 6.50; Calcd. for $C_{19}H_{25}O_4Br$: C, 57.43; H, 6.34).

Ozonization δ, δ-Dimethyl-γ-*iso*butenyl-δ-valerolactone (1.0 g.) dissolved in chloroform (20 ml.) was ozonized at 0°. The solvent was evaporated under reduced pressure and the residual ozonide was decomposed with water (40 ml.) and left standing overnight. From the distillate, acetone was isolated as 2,4-dinitrophenylhydrazone m.p. 126° (mixed). On concentration of the remaining solution, δ,δ-dimethyl-γ-carboxy-δ-valerolactone (0.7 g.) m.p. 175° (*Anal.* Found: C, 55.97; H, 7.09; Calcd. for $C_8H_{12}O_4$: C, 55.80; H, 7.03); equivalent weight by titration, Found 174.2 Calcd. for $C_7H_{11}O_2(CO_2H)$ 172.2 (yield 0.55 g.). For infra-red, see Fig. in the text. Ozonization of the both lactones from different sources **a)** and **b)** afforded the same lactonic acid as well as acetone. The lactonic acid was hydrolyzed with 0.35 N-ethanolic potassium hydroxide and the resulting hydroxy-acid, though not isolated crystalline, gave with Denige's reagent, a positive test for a tertiary hydroxyl group and a negative test with Nessler's reagent.

Acknowledgements The authors are indebted to Profs. S. Takei and M. Ohono of Kyoto University for their advice and also to Mr. K. Uyeyama, President of this Company, for his encouragement in the course of this work.

[Bull. Agr. Chem. Soc. Japan, Vol. 22, No. 3, p. 194~200, 1958]

Intracellular Lipase Formation by Washed Mycelium

Biochemical Studies on *Sclerotinia Libertiana*. Part 13

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Received November 26, 1957

Intracellular lipase of the fungus *Sclerotina Libertiana* Fcl. could be formed powerfully by washed mycelium during shaking in a plain buffer solution, just as well as in the case of shaking culture. Experiments showed revealed it to be favourable to set the mycelium in the experiment harvested at the end of its stationary phase of growth, and that the addition of various respiratory carbon sources had inhibiting effects, while several surface active agents and some enzyme preparations accelerating effects on the lipase formation. Also, the quality and the quantity of consumed cell-materials in the shaking experiment were investigated in relation to lipase formation.

INTRODUCTION

Several studies on the formation of lipase by microorganisms have been published in the past.(1)-(5) A.M. Stern(4) and his coworkers reported that the lipase activity of *Mucor mucedo* reached its maximum in only 9 days in its culture at 30°C, and that soy-bean oil was the most suitable carbon source, highly aerating conditions as on a shaking machine lead to a marked decrease in activity. I.I. Peters and F. E. Nelson(1) mentioned that in the case of *Mycotorula Lipolytica* lipase production was definitely restricted by aeration. In Japan, C. Matumura(5) stated that *Rhizopus* species excreted powerfully the exocellular lipase into the medium in its submerged culture at 30°C for 4 days. Comparing these works with that of other enzyme formation, it may be generally said that for the lipase formation it does not involved the adaptation, in its exact sense, but a comparatively long time is required in the culture, enzyme activity not appearing until the end of the stationary phase of growth and aeration has a retarding influence.

Studies on the formation of enzyme by washed mycelium of *Aspergillus* molds were made by H. Shiota(6,7) on protease, and by K. Tonomura and O. Tanabe(8) on amylase, also by J. Fukumoto and his coworkers(9) on the formation of bacterial amylase and proteinase by washed bacterial cell.

The results of these authors lead to the conclusion that the enzyme are not accumulated in the medium or within the cell while respiration or growth plays the leading role of metabolism in the organisms. Lately, B. Magasanik and his coworkers(10) have reported on the metabolic regulation of the enzyme biosynthesis in bacteria. It was said there that the "feedback" mechanisms by which intermediary metabolites can be controled their level in the cell, are concerned with the process involved in the formation of "adaptive" or "inducible" enzymes.

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* Osaka Bacteriological Research Institute Ltd.

1) I. I. Peters and F. E. Nelson, *J. Bact.*, **55**, 581 (1948).

2) J. J. Goodman, *Science*, **112**, 76 (1950).

3) C.V. Ramakrishnan and B. N. Banerjee, *Enzymologia*, **XV**, 33, 98 (1952).

4) A. M. Stern, J. Ordal and H. O. Halvorson, *J. Bact.*, **68**, 24 (1954).

5) C. Matumura, Meeting of Agr. Chem. Soc. Jap. (1956).

6) H. Shiota, *J. Agr. Chem. Soc.*, **30**, 724 (1956).

7) H. Shiota and T. Mine, *ibid.*, **31**, 173 (1957).

8) K. Tonomura and O. Tanabe, *J. Agr. Chem. Soc.*, **30**, 596, 598 (1956).

9) J. Fukumoto, T. Yamamoto and D. Turu, *J. Agr. Chem. Soc.*, **31**, 506 (1957).

10) B. Magasanik, F.C. Neidhardt and A. P. Levin, *Int. Symp. Enz. Chem.*, (1957) topic 3, 317.

With respect to the formation of lipase by *Sclerotinia* the original strain Ss was far superior to Sm, a mutant which does not form sclerotium, in bran Koji culture as reported in the previous paper.(11) Sclerotium itself had no lipase activity and so it would be assumed that a close relation may exist between the lipase formation and the physiological condition of the mycelium in the period of sclerotium formation. But lately it was recognized that the lipase was abundantly produced without forming sclerotium in the shaking culture. In this report, studies were made on the condition of lipase formation by washed mycelium obtained in shaking culture.

EXPERIMENTAL AND RESULTS

Method; The activities of lipase[3,12], lecithinase (phospholipase), acetylesterase and pectinesterase were measured by the method as reported in the previous paper[11] and given by the difference between the sample and the blank in terms of c.c.s. N/20 NaOH. The activity curve shown in Fig. 1 indicates that intracellular lipase activity is in direct proportion to the amount of mycelium within the limit of about 50 mg dry weight in these experimental conditions.

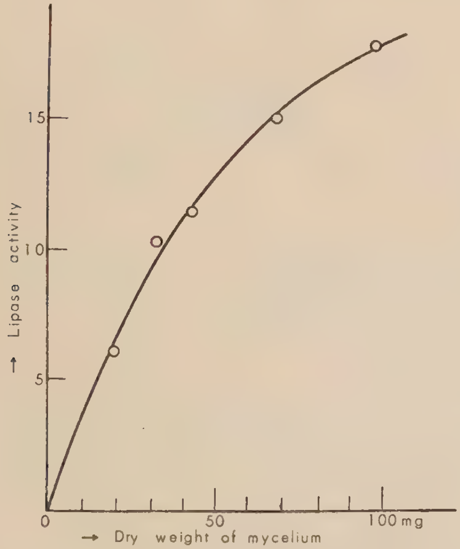

FIG. 1. Activity Curve Showing the Relation between the Intracellular Lipase Activity and the Amount of Mycelium obtained by Basal Medium.

The fungus *Sclerotinia* was inoculated in each 100 c.c. of the medium taken in a 500 c.c. shaking flask placed on a shaker of 7.5 cm in amplitude and 150 r.p.m. and incubated at 25°C. for a few days. And washed mycelium was prepared by harvesting at a time fit for the lipase formation and centrifuging and washing with 0.6% NaCl solution three times, then the mycelium, after resuspending in 0.6% saline or M/20 phosphate buffer solution (pH 6.0), was shaken again at 25°C. to measure the lipase activity produced progressively at appropriate intervals.

Shaking growing culture;

(a) Effect of the composition of medium. Using 10% decoction of wheat-bran as the basal medium, the effect of some carbon or nitrogen sources added to this on lipase formation was examined as shown in Table I.

As the results indicate, the basal medium to which only a small amount of $CaCO_3$ was added was found to be much favourable but the double diluent of this medium was more excellent for the lipase formation. When the pulverized bran-meal was used itself, instead of the decoction, the nutrient was most efficiently utilized even in a minimum quantity. The addition of any carbon or nitrogen source to the basal medium rather inhibited the formation of lipase.

(b) Relation of sugar-consumption, pH change and mycelial growth with the formation of lipase and other esterases.

As shown in Fig. 2, the time of appearance of the lipase activity of both strains Ss and Sm, differing from that of pectinesterase and acetylesterase, was recognized at the period when sugar in the medium had been consumed far after the growing phase. Also, the pH of the culture medium dropped for a while in proportion with sugar-consumption and afterwards rose progressively in parallel with the increase of lipase activity. On comparing Ss with Sm, the pH drop of Ss was larger than that of Sm, corresponding to higher lipase formation of the former than that of the latter.

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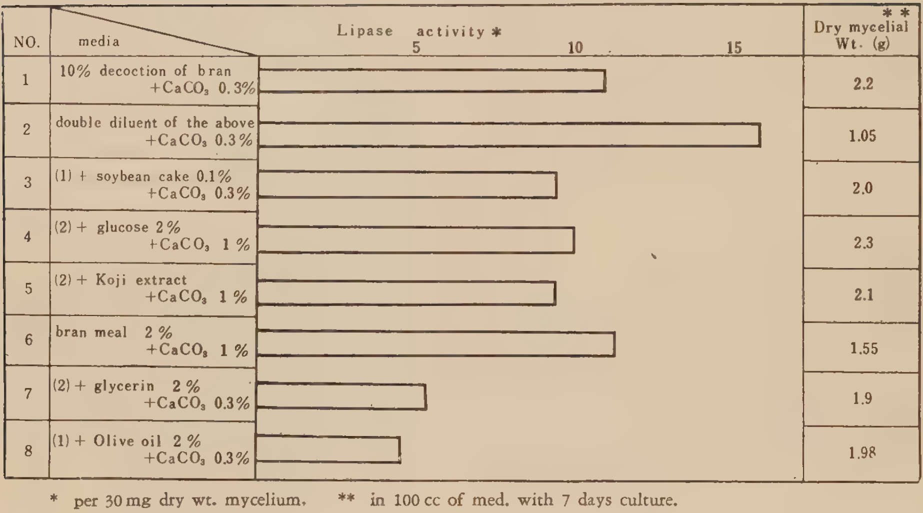
[11] Y. Satomura and S. Oi, *J. Agr. Chem. Soc.*, **31**, 202 (1957).
[12] J.V. Fiore and F. F. Nord, *Arch. Bioch. Biophys.*, **23**, 473 (1949).

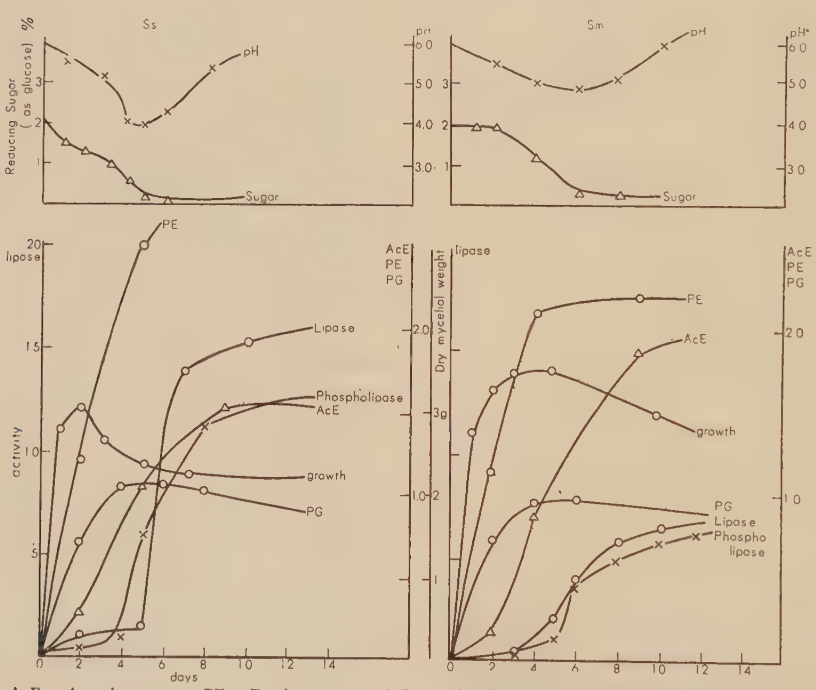
TABLE I

EFFECT OF COMPOSITION OF MEDIUM


Lipase activity *
5
10
15

NO.	media	Dry mycelial Wt. (g) **
1	10% decoction of bran +CaCO$_3$ 0.3%	2.2
2	double diluent of the above +CaCO$_3$ 0.3%	1.05
3	(1) + soybean cake 0.1% +CaCO$_3$ 0.3%	2.0
4	(2) + glucose 2% +CaCO$_3$ 1%	2.3
5	(2) + Koji extract +CaCO$_3$ 1%	2.1
6	bran meal 2% +CaCO$_3$ 1%	1.55
7	(2) + glycerin 2% +CaCO$_3$ 0.3%	1.9
8	(1) + Olive oil 2% +CaCO$_3$ 0.3%	1.98

* per 30 mg dry wt. mycelium. ** in 100 cc of med. with 7 days culture.

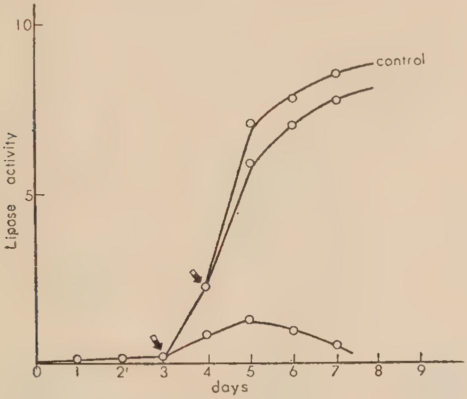


AcE: Acetylesterase PE: Pectinesterase PG: Polygalacturonase Media: Basal medium
10% decoction of bran
0.3% CaCO$_3$

FIG. 2. Relation of Sugar Consumption, pH Change and Mycelial Growth with the Formation of Lipase and other Esterases.

Shaking washed mycelium ;

(a) Influence of some carbon sources and specific inhibitors. The time suitable to harvest mycelium for the washed preparation was found to be that when the culture was 4 days' old, sugar in the medium had been consumed and the pH had fallen down to its minimum. The mycelium in that state of culture could form the lipase powerfully by shaking in plain buffer solution as well as in prolonged shaking culture as shown in Fig. 3. But the mycelium 3 days' old in culture was found to be very weak in ability to form lipase, so the washed mycelium used in the following experiment was prepared within a day after the consumption of sugar was recognized in the medium.

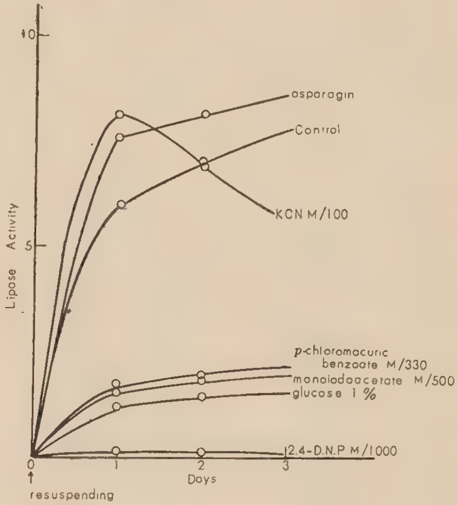


⬇; Washing and resuspending in buffer solution.
Control ; Prolonged shaking culture.

FIG. 3. Relationship between the Time to Harvest Mycelium to prepare the Washed Preparation and the Activity of Forming Lipase.

Influence of various respiratory carbon sources and specific inhibitors on the lipase formation by washed mycelium were examined as shown in Fig. 4. The relative activity was caluculated by comparing the maximum enzyme activity reached in 48 hrs. with the control. As shown in the results, all sugars added inhibited the lipase formation and initiated mycelial growth. Accordingly it is conceivable that the lipase forming system was suppressed by the brisk metabolic action coupled with the exogeneous respiration and the growth intiated by sugar supply. As the concentration of sugar was reduced, then its inhibitory effect also was reduced gradually, but it is worthy of note that the lipase formation was yet inhibited remarkably by such a low concentration as 0.1% of glucose. And it may be a characteristic of strain Ss that the effect of lactose is much greater than that of maltose. Ethylalcohol had also inhibitory effect on the enzyme formation and enhanced mycelial growth. Olive oil which can not be untilized well in growing medium had also no effect on lipase formation by washed mycelium.

2, 4-Dinitrophenol, *p*-chloromercuricbenzoate and monoiodoacetate all inhibited the lipase formation, then it can be considered that the phosphorylating process and the action of enzyme containing SH-group play a role in the synthesis of the enzyme protein as in the case of many other enzymes in living organism, whereas cyanide had rather a tendency to increase the lipase formation. A very small quantity of asparagine added also increased the lipase formation in some degree but the mechanism of the effect exerted to the

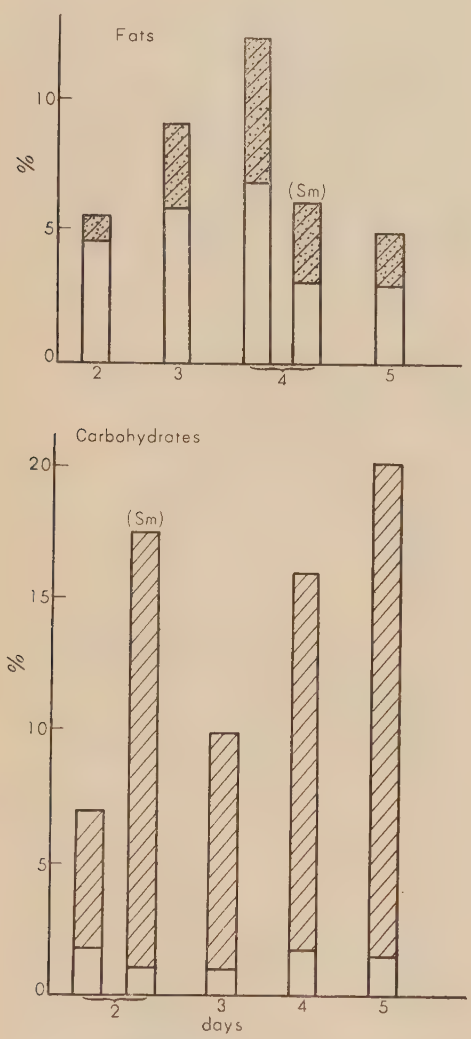


		Relative activity
Control	—	100
Glucose	2.0%	0
	1.0	23
	0.1	56
Maltose	1.4	75
Lactose	1.4	0
Olive oil	1.0	100
Ethanol	0.5	27
2-4 D. N. P.	M/1000	0
	M/5000	50
KCN	M/100	157
	M/500	135
p-Chloromacuric benzoate	M/330	26
	M/3000	30
$CH_2J \cdot COOH$	M/500	26
	M/1000	30
Asparagin	M/500	125

FIG. 4. Effect of Various Respiratory Carbon Sources and Specific Inhibitors.

cell is uncertain.

(b) Relation of the changes in quality and quantity of reserved cell-materials during shaking experiment with the lipase formation.



Fat { ☐ Free fat, directly extracted with ether in 6 hr.
▨ Bound fat, after hydrolysis of remainder of the above with 2 N HCl for 2 hr. again extracted with ether for 6 hr.

Carbohydrate { ☐ Reducing sugar (as glucose).
▨ Total sugar measured by the hydrolysis of mycelium with 1 N HCl for 2 hr.

FIG. 5. Changes of Carbohydrate and Fat in % of Dry Cell-material in the Course of Culture.

Since the lipase formation by washed mycelium is, as shown above, linked in many respects to the endogeneous respiration, then a clue to the explanation of the mechanism of lipase formation would be found by examining the change of reserved materials within the cell during the shaking. In Fig. 5. an examples of the change of carbohydrate and fat in percent of dry cell-material in the course of culture is given. The fat content increased at first with the lapse of days but decreased abruptly at the time when lipase formation was maximal. On the contrary, the carbohydrate content increased continuously with the age of culture, regardless of lipase formation. The accumulation of fat by Sm was smaller than that by Ss, but the accumulation of carbohydrate just reverse. Accordingly, the metabolism of fatty substance seems to be in a direct relation with the formation of lipase.

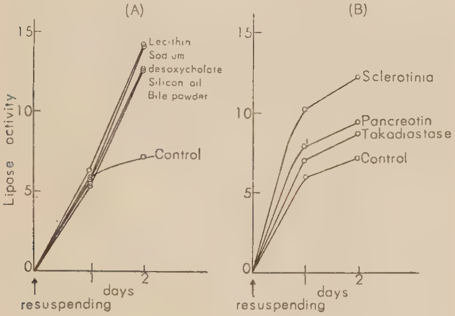
Table II shows the decrease in carbohydrate and fat content after shaking the mycelium, comparing with the lipase activity measured at the same time. As seen, the increase in lipase activity is quite proportional to the decrease in fat content, whilst it is independent to the change of carbohydrate. From these results, it would verified that the lipase formation is coupled with the metabolism of fatty substance.

TABLE II

DECREASE IN CARBOHYDRATE AND FAT, AND THE FORMATION OF LIPASE BY WASHED MYCELIUM DURING SHAKING IN BUFFER SOLN.

Days of pre-culture		2	6	8
Times of shaking the washed mycelium (hr.)		48	48	48
Lipase activity appeared		2	7	4
Decrease in fat (%)	Free fat	78.5	83.8	30.3
	Bound fat	17.5	83.2	70.0
Decrease in carbohydrate (%)	Reducing sugar	70.8	45.8	84.5
	Total sugar	73.8	24.4	83.4

(c) Influence of several surface active agents and some enzyme preparations. In shaking culture, it was found microscopically that the mycelium gradually became segmented to oidien or chlamidosporen and seemed to be changing to pseudoparenchymatic cells, as in the case of sclerotium formation, particularly when the lipase was abundantly produced. It was then supposed that the intracellular lipase may be formed more powerfully by promoting the catabolic action involved in the above process of changing in cell structure at the end of culture. From such a view point, the effects of various surface active agents and some enzyme preparation added to the washed mycelium were examined. Thereby, as shown in Fig. 6, the additions of a small amount of surface active agents such as, bile-powder, desoxycholate, silicon oil and lecithin, and some enzyme samples such as, Takadiastase, Pancreatine and the extract of dry mycelial powder of *Sclerotinia* bran culture, were all found to promote, more or less, the formation of lipase. It is noteworthy that the extract of *Sclerotinia* itself is especially more effective than other enzyme samples. The surface active agents and enzyme here used had, of course, no influences in the measurement of lipase activity.



(A) Surface Active Agents

	Conc. %	Relative activity
Control		100
Lecithin	0.5	200
Bile powder	0.05	180
Sodium desoxycholate	M/500	200
Silicon oil	0.05	180

(B) Enzymes

	Conc. %	Relative activity
Control		100
Pancreatin	0.05	134
Takadiastase	0.1	120
Sclerotinia dry mycelial powder	0.1	180

FIG. 6. Effect of Surface Active Agents and Enzyme Preparations for Lipase formation.

DISCUSSION

Though the lipase formation in washed mycelium was proved to proceed in a rate quite proportional to the consumption of fatty substance reserved in the cell, the important point to consider, in this respect, would be the mode of metabolic regulation of the reserved cell-materials. In fact, the mutant strain Sm, which has a high content of carbohydrate as reserve material, could not produce as much lipase as the original strain. The carbohydrate metabolism, predominated in this case, have suppressed the fatty one and hence lipase formation.

As the metabolic products of *Sclerotinia* in liquid surface culture, M. Kitahara(13) determined ethylalcohol, acetaldehyde, and as principal volatile or non-volatile organic acid, acetic acid and oxalic acid. Then, the fungus *Sclerotinia* seemed to have a weak fermenting activity. In the above described experiments, since the notable accumulation of any organic acid had not been detected, the cause of lowering pH might be ascribed to the transformation of phosphate compound within the cell and the release of inorganic phosphate into the medium. But when $CaCO_3$ was not added to the basal medium, a considerable quantity of organic acid was found to accumulate in the medium at an early period, i.e. when the age of shaking culture was about 2 days' old, although it soon disappeared in the course of culture. In the case of bran culture, the period of lipase formation follows that of sclerotium formation, and it may be considered that the liberation of inorganic phosphate would not be due to simple autolysis, but to the utilization and the decomposition of energy-rich phosphate bonds in the exhaustion process of initially accumulated alcohol or organic acids or in the process of sclerotium formation. Whereas, with Sm, as the accumulation of any organic acid or the liberation of inorganic phosphate had not been recognized, it was therefore considered that Sm also differs from the original strain, Ss, in some way of carbohydrate metabolism.

Recently, the inhibitory effect of glucose on the formation of inducible(10) or non-inducible(14) bacterial enzyme was demonstrated and the physiological significance of the effect of glucose and other carbon compound to comprehend the process of enzyme biosyntheses(10) has been investigated. The

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13) M. Kitahara and Y. Takeuchi, *Res. Bull. Coll. Agr. Gifu Univ.*, No. 1, 86 (1951), No. 2, 71 (1953).

14) J. Fukumoto, T. Yamamoto and D. Tutu, *Nature*, **180**, 438 (1957).

observed glucose inhibition on the lipase formation in the washed mycelium experiments could be reasonably explained by the "feedback" mechanism, because glucose and fatty substances are known to be metabolized in quite a close relation.

Contrary to previous reports that aerating condition exerted the retarding effect on the lipase formation of various microorganisms(1),(4), the shaking condition seemed to have no inhibiting effect in the case of experiments conducted with *Sclerotinia* fungus as described above.

It is noteworthy that the cyanide has no influences upon the formation of lipase by the fungus, occurring on the expense of reserved fat consumed in the endogeneous respiration. Further investigation on this subject is necessary.

As to the effects of surface active agents on cellular metabolism, it had so far been known that anionic detergents, such as bile salts, cause lysis by attacking the cell-wall concurrently promoting the action of the autolytic enzyme system of many organisms. Lately, W. M. Armstrong(15) has shown the inhibitory effect of cationic detergents on the production of acid and CO_2 by yeast, and in his data, some of the anionic detergents were shown to be not completely inhibitory, but rather stimulatory for the metabolic action. The increasing effect of surface active agents and some enzyme preparations on the lipase formation by *Sclerotinia* is eventually considered to be concerned with the metabolic system linked to the enzyme formation.

SUMMARY

1. The mycelium of *Sclerotinia* harvested within a day after sugar was exhausted in the medium of its shaking culture, which was found to be the most favourable physiological condition for preparing the washed mycelium for the experiment of lipase formation.

2. In the experiment with washed mycelium, although various sugars and other carbon sources caused remarkable inhibition to the lipase formation olive oil had no influence. Cyanide had no effect, 2,4-dinitrophenol, *p*-chloromercuric benzoate and monoiodoacetate displayed inhibitory action. Surface active agents such as bile-powder, silicon oil and lecithin, and some enzyme preparations, particularly the extract of dry mycelial powder of *Sclerotinia* itself accelerated enzyme formation.

3. The rate of lipase formation proceeded in proportional to that of fat consumption of the mycelium.

The authors wish to express their thanks to Prof. I. Yamasaki for his careful revision, Prof. J. Fukumoto for his encouragement and to Mr. N. Yoshizu, Director of Osaka Bacteriological Research Institute for his helpful support. They are also indebted to Mr. K. Tomoda for his assistance in part of the experiments.

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15) W. M. Armstrong, *Arch. Bioch. Biophys.*, 71, 137 (1957).

Short Communications

The Essential Oils of *Mentha rotundifolia* of European Origin

(Studies on the Essential Oil of *Mentha rotundifolia*, Part IV)

Sir:

Rotundifolone $C_{10}H_{14}O_2$, the principal component of *Mentha rotundifolia* oil in Japan, has been established to be 1-methyl-4-isopropylidene-1, 2-epoxy-cyclohexanone-3 (1, 2-epoxy-piperitenone) by our previous reports.1) Reitsema2) isolated the same ketone from the oil of American *M. rotundifolia* and independently gave the same structure as ours via a different way. However, Rovesti,3) Schimmel group4) and Costa5) did not find any α,β-unsaturated ketone in their European *rotundifolia* oils. On the other hand, pulegone was found in the oils of Argentina6) and Morocco.7) Our Japanese *M. rotundifolia*, though is not native in this country, was introduced as a sample from an unknown foreign country about twenty years ago. According to Murray,8) the American strain is shown to be of European origin.

From the above-mentioned facts, *M. rotundifolia* will presumablly be native in Europe and may at least be divided into two groups; one contains rotundifolone, and the other does not. In order to elucidate this problem, we have collected various strains of *M. rotundifolia* of European origin, which are shown in the following Table I:

TABLE I

1. France: Jardin Botanique de Toulouse, 1955.
2. Jugoslavija: Hortus Botanicus Univ. Ljubljana, 1955.
3. Bergie: Plantentuin der Rijiksuniversiteit te Gent, 1955.
4. Italy: Instituto ed Ort Botanico della Univ. di Roma, 1955.
5. Polgne: Institut Scientique National des Drougues végètales, Poznan, 1956.
6. Portugal: Estaçaõ Agronomica Nacional Sacavém, 1956.

Since the genus *Mentha* is taxonomically exceedingly complicated, the seeds of European origin mentioned above were imported and cultivated in Okayama, Japan. The somatic chromosome numbers of each strain were determined to be 24 by the method reported previously.9) The essential oils of these strains were found to have the following physico-chemical properties;

TABLE II

Origin	λ_{max}	Optical density (10 γ/cc)	d_4^{20}	n_D^{20}	$[\alpha]_D^{20}$
France	235mμ	0.240	0.934	1.4833	− 3.27°
Jugoslavia	260	0.481	1.024	1.5010	+148.33
Bergium	260	0.456	1.015	1.4962	+121.92
Italy	260	0.478	1.012	1.5000	+157.50
Poland	260	0.425	1.008	1.4875	+ 95.16
Portugal	260	0.400	0.993	1.4871	+ 72.73

As shown in the above table, the oils of five strains except the French strain were highly dextro-rotatory and showed strong absorption at 260 mμ. The presence of rotundifolone was also proved by semicarbazone and iso-rotundifolone (diosphenolene) formation1). These facts indicate that most of European *Mentha rotundifolia* are completely identical with those of Japan and America. But the French oil shows different aspects, which gave 2,4-dinitro-phenylhydrazone melting at 157° as a red-orange crystal. The detailed properties of this oil will be reported in the near future.

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Received March 19, 1958

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1) S. Shimizu, *Journal of the Faculty of Agric., Shinsyu Univ.*, No. **6**, 1-39 (1956); This Bulletin, **21**, 107 (1957).

2) R. H. Reitsema, *J. Am. Chem. Soc.*, **78**, 5022 (1956).

3) R. Rovesti, *Profumi italici*, **3**, 178 (1925).

4) *Ber. Schimmel Co.*, April 80 (1907).

5) A. F. Costa, *Bol. Escola Farm. Univ. Coimbra*, **5**, 90 (1945).

6) G. A. Fester, E. A. Martinuzzi, J. A. Retamer and I. A. Ricciardi, *Biol. de la Academia National de Ciencias*, **39**, 386 (1956); *C. A.*, 7658 (1957).

7) J. Gattefosse and G. Igolen, *Ber. Schimmel Co.*, 70 (1950); *C. A.*, 7193. (1949),

8) Private communication from Dr. V. J. Varnis, A. M. Todd Co., Oct. 10, 1957.

9) N. Ikeda and S. Udo, *Scientific Reports of Faculty of Agric. Okayama Univ.*, **4**, 43 (1954).

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[Bull. Agr. Chem. Soc. Japan, Vol. 22, No. 3 p. 202, 1958]

The Biological Activity of Some Homologs of Mevalonic Acid

Sir:

Mevalonic acid (MVA), β-hydroxy-β-methyl-δ-valerolactone, was shown by Wolf, et al.1) to be a new acetate-replacing and growth factor for lactobacilli. At about the same time, G. Tamura isolated hiochic acid independently, indispensable for the growth of *Lactobacillus homohiochii* and *L. heterohiochii*,2) and confirmed its identity with MVA.3) Recently, S. Tamura and Takai4) reported on the synthetic preparation of β-ethyl-β-hydroxy-δ-valerolactone (I), α,β-dimethyl-β-hydroxy-δ-valerolactone (II) and β,γ-dimethyl-β-hydroxy-δ-valerolactone (III) and preliminarily pointed out the inhibitory action of II against *Lactobacillus heterohiochii* in the presence of MVA.

In this paper we wish to describe the biological activity of some MVA homologs. The compounds tested were as follows: I, II, III, α-ethyl-β-hydroxy-β-methyl-δ-valerolactone (IV), b.p. 136°/3 mm, β-hydroxy-α,α,β-trimethyl-δ-valerolactone (V), b.p. 145–147°/3 mm, and α-*n*-butyl-β-hydroxy-β-methyl-δ-valerolactone (VI),5) b.p. 151–155°/1mm. The latter three are new compounds which were prepared by the method almost similar to that for II, and their structures were confirmed by microanalyse and infrared spectra. Each of the six homologs did not show activity as a growth factor for *Lactobacillus heterohiochii* when added to the broth medium of G. Tamura6) in place of MVA. This fact indirectly suggests that MVA must be a precursor for the isoprene unit. When these compounds were added at the concentration of 100γ/ml to the medium containing 1γ/ml of DL-MVA, II or IV completely inhibited the growth of the above lactobacillus while the others showed no inhibition. The minimal concentration of DL-MVA necessary to support growth in the presence of 100γ/ml of II or IV is 4γ/ml or 3γ/ml respectively. From these results it may be concluded that only the MVA homologs which have a small alkyl group such as the methyl or ethyl group on their α-carbon atoms can be incorporated into an isoprenoid-biosynthetic systems thus competitively inhibiting the utilization of MVA. At any concentrations, II and IV could not inhibit the growth of the lactobacillus which did not require MVA. Moreover, even at the concentration of 100γ/ml, II or IV did not inhibit the growth of *Bacillus subtilis* P.C.I. 219, *Escherichia coli*, *Staphylococcus aureus* 209 P, *Corynebacterium xeroces*, *Sarcina lutea*, *Candida albicans*, *Tolura utilis* and *Saccharomyces cerevisiae*.

We wish to express our sincerest thanks to Prof. Y. Sumiki for his guidance and encouragement throughout the course of these experiments.

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Received March 31, 1958

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1) D. Wolf, C. Hoffman, P. Aldrich, H. Skeggs, L. Wright and K. Folkers, *J. Am. Chem. Soc.*, **78**, 4499 (1956).

2) G. Tamura, *J. Gener. Appl. Microb.*, **2**, 431 (1956).

3) G. Tamura, This Bulletin, **21**, 202 (1957).

4) S. Tamura and M. Takai, *ibid.*, **21**, 394 (1957).

5) These six compounds are the respective mixtures of stereoisomers.

6) G. Tamura, Nippon Nôgei-Kagaku Kaishi (in press).

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[Bull. Agr. Chem. Soc. Japan, Vol. 22, No. 3, p. 203~204, 1958]

Studies on Lignin

Part V. A Reexamination of Hydrogen Sulfide Cooking of Vanillyl Alcohol, and Conversion of Vanillyl Disulfide into Vanillyl Monosulfide

Sir:

In the course of studies conducted on the reaction of lignin in the sulfate process, Enkvist and Moilanen found that vanillyl alcohol, which had already been known as an important lignin model compound in the sulfite process, would form vanillyl disulfide upon cooking with a hydrogen sulfide solution.(1) Later, contrary to this finding, Mikawa obtained a mixture of vanillyl monosulfide and vanillyl disulfide from the result of a similar experiment.(2) Mikawa, to account for this discrepancy, has discussed the amount of oxygen in ampoule, and Enkvist, in his personal letter to Mikawa, has pointed out the difference of pH in cooking solutions and the concentration of hydrogen sulfide.(2)

In an attempt to find out the factors which determine the formation of vanillyl disulfide or vanillyl monosulfide, a series of experiments on the hydrogen sulfide cooking of vanillyl alcohol at various pH was carried out. Result of cookings (100°, 7 hours) in acetate buffer solutions is shown in Table I. On the basis of these data, it seemed reasonable to assume that the determining factor is the pH of the cooking solution. Although the oxygen contents of the cooking solutions were not determined, solutions slightly acidic gave vanillyl disulfide even after the expulsion of oxygen by bubbling through hydrogen sulfide. The reaction products, after one recrystallization from toluene, showed the same melting points as vanillyl disulfide or vanillyl monosulfide, and were identified by mixed melting point determinations with authentic specimens. However, in case of cooking with phosphate buffer solutions in which hydrogen sulfide was bubbled through, the effect of pH was not so distinct as in the case of acetate buffer; the main product was vanillyl monosulfide even in acidic solutions. Gierer reported that *p*-hydroxybenzyl alcohols yielded monosulfides when cooked with a sodium hydrosulfide solution of pH 9, but unfortunately no description was given on the conditions by which disulfide was formed.(3) Some unknown factors on the reaction mechanism of disulfide- and monosulfide-formation may still exist.

TABLE I

Cooking solution	pH Prior to cooking	pH After cooking	Product
$Na_2S \cdot 9H_2O$ 1.7g + AcOH 0.6cc/50cc	6.4	9.7	M. S.
〃 + 〃 ~~0.9~~ 0.7	6.3	7.3	M. S.
〃 + 〃 0.8	5.7	6.5	D. S.
〃 + 〃 1.0	5.0	5.4	D. S.

According to Schöberl, disulfide groups were converted into mercaptans and sulfenic acids, of which the latter were further decomposed to carbonyl groups upon heating with alkaline solution.(4) Enkvist and Moilanen supported this finding after they had obtained vanillin by alkali treatment of vanillyl disulfide.(1) Also, on the other hand according to Schöberl, cystine (disulfide) in wool upon soda treatment is reported to yield lanthionine (monosulfide).(5) It therefore,

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1) T. Enkvist and M. Moilanen, *Svensk Papperstidn.*, **52**, 183 (1949).
2) H. Mikawa, *Bull. Chem. Soc. Japan*, **27**, 50 (1954).
3) J. Gierer and B. Alfredsson, *Acta Chem. Scand.*, **11**, 1516 (1957).
4) A. Schöberl and H. Eck, *Ann.*, **522**, 97 (1936).
5) A. Schöberl, *Chem. Ber.*, **76**, 970 (1943).

seems to be of interest to investigate the possibility of conversion of vanillyl disulfide into monosulfide, in view of the fact that while the hydrogen sulfide lignin obtained from hydrogen sulfide cooking of wood at neutral pH, contained disulfide- and monosulfide-sulfur only monosulfide-sulfur was found in thiolignin obtained from sodium hydrosulfide cooking treatment.(6) Cooking of vanillyl disulfide with a hydrogen sulfide solution of pH 9 has been found to yield vanillyl monosulfide. Whereas, on the other hand, vanillyl disulfide was recovered in an unchanged state by cooking with buffer solutions of the same pH containing no hydrogen sulfide. However, when the pH of borate buffer was increased to 10 or 11, vanillyl disulfide was converted into monosulfide also being accompanied by the formation of vanillin. Based on these findings the following scheme might be proposed tentatively as:

$$2\,\mathrm{R{-}CH_2SSCH_2{-}R} + 2\,\mathrm{H_2O} \longrightarrow \mathrm{R{-}CH_2SCH_2{-}R} + 2\,\mathrm{R{-}CHO} + 3\,\mathrm{H_2S}$$

(R=4-hydroxy-3-methoxyphenyl)

The determination of the reaction products, however, revealed that the amount of vanillin and hydrogen sulfide thus formed is about one half of the calculated value. Further, the amount of vanillyl monosulfide (total weight of insoluble material, of which one recrystallization from toluene yielded almost pure monosulfide) was slightly larger than expected, after cooking for seven hours at 100°, the over all recovery of aromatic nucleus being about eighty per cent.

As intermediate products resulting from the process of cystine to lanthionine, Schöberl presented dehydroalanine (α-aminoacrylic acid), cysteine and serine.(7) (8) In our case these products correspond to quinone methide, vanillyl mercaptan and vanillyl alcohol, respectively.

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Received April 3, 1958

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(6) T. Enkvist and E. Hägglund, *Festskrift J. Arvid Hedvall*, **1948**, 149.

(7) A. Schöberl and A. Wagner, *Angew. Chem.*, **68**, 215 (1956).

(8) cf H. Musso, *ibid.*, **68**, 313 (1956).

Synopses of the Articles printed in NIPPON NÔGEI-KAGAKU KAISHI
(in Japanese)
Vol. 32, No. 3 (1958)

The Studies on the Milling Quality of Domestic Wheat. Part II. On the Quality of Wheat and Wheat Flour harvested at Various Stages of Maturity. (2) Vitrous Domestic Wheat. (p. *163～167*)

By Tokuji WATANABE, Osamu WATANABE, Takashi YASUNAGA and Mitsuo UEMURA

(*Food Research Institute, Ministry of Agriculture and Forestry*),

By Tomoko HORIGUCHI

(*Tokyo Home Economic College*)

Following the mealy domestic wheat reported previously, the vitrous wheat was harvested at various stages of maturity. The characteristics of wheat flour each stage were examined to determine the stage most at suitable for harvesting from the view point of its utilization. The synthesis of gluten in wheat grain completes fairly later than starch. But the characteristics of dough of wheat flour tested by Brabender dough testing machine are already fairly good in early stages such as strong flour. The doughs of wheat flour in slater periods tend to be weaker. The starch granules of the flour are less damaged as wheat matures, because the wheat grain is more mealy in later stages than in early stages. It is therefore undesirable to harvest vitrous domestic wheat at the period of over-maturity.

The Influence of Feed and Feeding upon the Ruminal Gas Formation. Part III. Molasses-Urea Feeding. (1) (p. *167～171*)

By Tatsuro MATSUMOTO, Masayuki TAKAHASHI, Tetsuro KOMIYAMA and Masayuki YAMAMOTO

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Average variations of rumen gas compositions and rumen liquor properties, following molasses-urea and hay feeding, were measured. Samples of rumen gases were collected immediately before and at two, four, six and eight hour-periods after feeding. The contents of carbon dioxide, methane, hydrogen, oxygen and nitrogen were determined. Rumen liquors were gathered at two, four and a half, and seven hour-periods after feeding. The concentrations of V.F.A., ammonia-nitrogen and sugars were measured. All measurings were repeated three times.

The Influence of Feed and Feeding upon the Ruminal Gas Formation. Part IV. Molasses-Urea Feeding. (2) (p. *171～174*)

By Tatsuro MATSUMOTO, Masayuki TAKAHASHI, Tetsuro KOMIYAMA and Masayuki YAMAMOTO

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Increments of V.F.A. and ammonia-nitrogen, decrements of sugars, in the rumen liquor of goat, were estimated at ninety minutes after molasses-urea feeding. In the next experiment, chemical pure glucose and urea were injected into the rumen, and the disappearance of glucose and urea, production of V.F.A. and ammonia-nitrogen, were compared with the results of molasses-urea feeding. Changes of rumen gas compositions after glucose-urea injection were also examined.

Chemical Studies on Hair. Part IV. On the Mechanism of Concentrated Hydrogen Peroxide to Hair. (p. *175～177*)

By Masaru FUNATSU, Gunki FUNATSU, Yasuhiro IMAMARU and Takeshi NAGAOKA

(*Biochemical Laboratory, Faculty of Agriculture, Kyushu University*)

A study was made on the reaction of concentrated hydrogen peroxide to human dark hair. When hair was treated with 30% H_2O_2 (pH 1.63) at 30°C, the pH of H_2O_2 solution first arised, and there after gradually fell remaining constant (pH 1.0) after 40 hours. On the other hand, the nitrogen in the solution was resolved slightly within 24 hours, and afterwards increased remarkably. Although most of the nitrogen was due to hair peptide nitrogen, amino acids, such as the following were liberated ; Cys. a., Asp., Glu., Leu., Val., Ser., Thr. and Tyr.. Furthermore, it was found that the amino acids which were oxidized by H_2O_2 under this condition were sulfur containing amino acids and tryptophane. In the case of sulfur containing amino acids, $-SO_3H$ was resulted.

From the facts that the molecular weight of H_2O_2-treated hair was about 30,000 and that the hair was not greatly degradated*, we suggestedt hat the liberadet peptides and amino acids would not only be produced by the action of H_2O_2 oxidation, but also by the hydrolytic action of the $-SO_3H$ which was introduced into hair by H_2O_2 oxidation.

* G. Funatsu and M. Funatsu; *J. Agr. Chem. Soc. Japan*, **31**, 874 (1957).

Studies on the Nutritive Value of the Carbohydrate in Shiitake. (p. *178~181*)

By Yasuto WATANABE

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This paper is concerned with the fractionation of hemicelluloses of Shiitake (*Cortinellus Shiitake*) by dilute alkali, the hydrolysis of these fractions by acids or enzymes, the detection of their components, and their assimilabilities by rats.

The hemicelluloses were separated into three fractions according to their solubilities in 3% sodium hydroxide. Hemicellulose A, cold alkali-soluble fraction; B, hot alkali soluble-fraction; and C, hot alkali insoluble-fraction were obtained from dried Shiitake, in percentages of 13.6, 3.3, and 26.9, respectively.

The hydrolysis-degree by mineral acids or enzymes of Takadiastase or the extract of snail alimentary canal differed in each fraction. In the sulfuric acid hydrolysates of each fraction, the following substances were detected by means of paper chromatography and osazone test: glucose, mannose, xylose, and uronic acid in A; glucose, xylose, and uronic acid in B; and glucose and xylose in C.

By comparing the amount of glycogen accumulated in the bodies of adult rats the administration of each material, the relative assimilabilities of each hemicellulose preparation were in comparison with starch, found as follows: starch 100; A 70; B 34; and C 10. The increase of blood sugar by the administration of A was nearly equal to that of starch, but that of C was negligible, and that of B was moderate.

On the Migration Distance in Paper-electrophoresis. Part II. Analysis of the Migration Distance of Amino Acids. (1) (p. *181~186*)

By Kiyoshi SAKAMOTO and Kinuko SAITO

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The authors examined the migration distance of amino acids in phosphate buffer in the apparatus where evaporation from the surface of filter paper kept horizontal was admitted. The apparent migration distance from the original point at the time of t, MD', the net-initial velocity, V_0 and the distance of the stationary point, MD'_s were found to be approximately calculable in the following formulae.

$$MD'=MD'_1\cdot\frac{(a'/a)^n-1}{a'/a-1}$$

$$V_0=\frac{(MD'_1-MD'_{1dex})\cdot\log a'/a}{a'/a-1}$$

$$MD'_s=\frac{-MD'_1}{a'/a-1}$$

In the above formulae, MD'_1 and MD'_{1dex} represent the apparent migration distance of amino acids and dextran respectively from the original point at unit time of t_1 and a'/a the decrement ratio of migration velocity of the migrants at an interval of t_1, n, the t/t_1.

On the Migration Distance in Paper-electrophoresis. Part III. Analysis of the Migration Distance of Amino Acids. (2) (p. *186~188*)

By Kiyoshi SAKAMOTO and Kinuko SAITO

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The net-initial volocities $V_0\mathrm{cm}^2\cdot\mathrm{volt}^{-1}\cdot\mathrm{sec}^{-1}$ of arginine, lysine, histidine, glutamic acid and aspartic acid in phosphate buffers of several pH (8.0, 7.3, 6.8, 6.5, 6.0, 5.0) in the apparatus where evaporation from the surface of filter paper kept horizontal was admitted, were calculated by the following formula. Then the curves representing the relation between pH and migration velocities were prepared.

$$V_0=\frac{(MD'-MD'_{dex})\cdot\log a'/a}{a'/a-1}$$

In the above formula, MD' and MD'_{dex} represent the migration distance of amino acids and dextran respectively from the original point at the time of t and a'/a the decrement ratio of migration velocities of the migrants at an interval of t. The curves obtained above closely resembled those obtained by measuring the migration velocities in the filter paper clamped between glass plates. The migration velocities of neutral amino acids in the above pH of phosphate buffer were too small to obtain their respective curves.

Studies on Microbiological Assay of Vitamins and Amino Acids by the Pulp Disc Method. Part V. On the Rapid Determination of Vitamins and Amino Acids by the Pulp Disc Method. (p. *189~192*)

By Hisao KOJIMA and Yutaka MATSUYA

(*Department of Agricultural Chemistry, Faculty of Agriculture, Tohoku University*)

The procedure condition for the rapid determination of vitamins and amino acids was especially examined in respect to the relation between inoculum size and growth-zone. In the assay of several vitamins and amino acids, size and nature of the growth-zone formed were observed in detail along with the incubation period. As a result, shortening of the incubation-time for determination was successfully obtained. Further simplification of this method was also attempted.

Studies on the Inhibitory Factors in Acetone-Butanol Fermentation. Part III. Investigation on the Contaminated Bacteria. (p. *193~197*)

By Shigeru NEMOTO (*Gōdō-Shusei, Inc.*)

The author investigated on the contaminants which are considered to cause the abnormal fermentation.

The mashes which were extracted from the fermenter into the flask before the inoculation of butanol bacillus were carried out on Acetone-Butanol fermentation, though in a sluggish process. In process of the growth of the bacilli in the active culture and the 5L culture, a long linkage of the bacilli appeared.

The author then isolated the contaminants from fermented mashes, active cultures and sand cultures, and then classified them from various sugars by their acid productivity. One strain of coccus (No. 1) and four strains of bacilli (No. 2, No. 4, No. 6 and No. 12) were obtained.

As the results of mixed culture of butanol bacillus with the contaminants, it was found that this sluggishness of fermentation was caused by No. 1 and No. 2. Also the cultivations of butanol bacillus with the filtrates of contaminants filtrated through the Chamberlain, showed the same results as above. Contaminants No. 2 and No. 4 produced a certain amount of Acetoin which was included in the analytical value of Acetone.

From those investigations, all phenomena observed in this abnormal fermentation can be explained by the fact that this abnormal fermentation is caused by contaminant No. 2.

Studies on the Inhibitory Factors in Acetone-Butanol Fermentation. Part IV. Counter-Measures for Abnormal Fermentation. (p. *197~201*)

By Shigeru NEMOTO (*Gōdō-Shusei, Inc.*)

After researches on the counter-measures against the abnormality in this fermentation, the following results were obtained.

A concentration of Acetone or Butanol (0.1%) was inefficient sterillizer, and when the pH of mashes came below 4.4, growth of contaminants was checked. As most of the contaminants belong to aerobic bacilli, oil film prevention from air and the substitution of nitrogen gas for air was to some extent efficient. However, both methods were found difficult to be carried out in a large scale.

Bacillus No. 6 was efficient to some extent for preventation of harm caused by bacillus No. 2 to the growth of Butanol bacillus. Addition of Butanol bacillus to the mixed culture with bacillus No. 2, $(NH_4)_2SO_4$, K_2HPO_4, vitamin B_1, or Glutenmeal, were helpful in normal fermentation.

After investigating the characteristic of the above contaminants, the author has improved the methods previously employed in this factory for the culture of Butanol bacillus, and sterilized all equipments of the factory with formalin upon suspending the operation.

Polarographic Catalytic Waves of Proteins by Tris-(ethylenediamine)-cobalt (III) Complex. (p. *201~206*)

By Masahide ITO (*Mie Prefectural University*)

The polarographic effects of proteins were investigated in ammonia-ammonium buffer, using tris-(ethylenediamine)-cobalt (III) chloride $Co(en)_3Cl_3$. In this medium, lysozyme, ovalbumin and β-amylase gave a single wave respectively, which did not coincide with the protein double waves found by Brdička (1933) in ammoniacal solutions of hexammine-cobalt (III) chloride. The shapes of the waves of each protein were not identical.

The dependence of the wave-heights on the concentration of protein, $Co(en)_3Cl_3$, NH_4Cl and NH_3 were also studied, from which similar results were obtained such as in the case of the protein double waves, with the exception of NH_3 concentration. The concentration of NH_3 had only little effect on the wave-heights.

The influence of temperature on the wave-heights varied with each protein.

Consequently, comparing these polarographic behaviours with those of the protein double waves, it may be concluded that these single waves are the catalytic protein waves, and that such differences in the behaviours of protein waves, are probably based on the differences of the structure and chemical properties of two cobalt complexes.

to a certain period of the experiment, almost without any degree of self-digestion in the enzyme protein, while reactivation of the enzyme by the addition of zinc salts always accompanied a certain degree of self-digestion.

Studies on the Pyruvate and Carbohydrate Metabolisms by Lactic Acid Bacteria. Part VII.

Dismutation System of Sugars and Pyruvate by *L. brevis*. (p. *236～238*)

By Kei YAMANAKA
(*Kagawa Agricultural College*)

By the dried cells of *L. brevis*, ATCC 8281, a dismutation reaction between pyruvate and gluconate was demonstrated. In this reaction, pyruvate is reduced to lactate and gluconate is converted to lactate, acetate and carbon dioxide. The ratios of pyruvate removed, carbon dioxide evolved and of lactate formed were nearly 1 : 1 : 2. Furthermore, Bial's reaction of this incubation mixture was positive. Similar results were also obtained with glucose, fructose and maltose, but no evidence of Bial's reaction was given with mannose, galactose, sucrose, lactose or xylose.

Studies on the Pyruvate and Carbohydrate Metabolisms by Lactic Acid Bacteria. Part VIII.

Formation of Pentose Phosphate by the Hetero-type of Lactic Acid Bacteria. (p. *239～243*)

By Kei YAMANAKA
(*Kagawa Agricultural College*)

On the dismutation system between pyruvate and sugars by hetero-type of lactic acid bacteria, detection of pentose was carried out. Four strains of bacteria, *L. fermentum*, *Leuc. mesenteroides*, *L. brevis* and *L. pentoaceticus* were used.

Each incubation mixtures gave a positive Bial's reaction ($\lambda_{\mathrm{max}}=670\,\mathrm{m}\mu$), crysteine-sulfuric acid reaction ($\lambda_{\mathrm{max}}=390\,\mathrm{m}\mu$), α-naphthol reaction ($\lambda_{\mathrm{max}}=550\mathrm{m}\mu$), and cysteine-carbazole reaction ($\lambda_{\mathrm{max}}=540\mathrm{m}\mu$). This substance was obtained in barium soluble and alcohol insoluble fractions, and its barium salt was stable for bromine oxidation.

These results indicated that ketopentose phosphate was formed in this dismutation reaction.

Rheological Studies on Potato Starch. Part III.

Effects of Metal Ions on Potato Starch. (p. *243～250*)

By Shizue TAKAHASHI and Yoshijiro KIHARA
(*Laboratory of Food Chemistry, Faculty of Home Ecnomics, Ochanomizu University, Tokyo*)

The effects of metal ions on the rheological properties of potato starch paste and of starch during ordinary storage have been examined by both the static viscoelasticity measurement and the Brabender amylograph. Phosphorus and iron contents and spectroscopic reflective index have also been determined.

The rheological properties of starches treated with 0.5N solutions of metal chlorides have been proved to be considerably affected. Treatment with ferric chloride solution exerts a heavy influence on the spectroscopic reflective index and on color analysis values, and swelling property was also extremely inhibited owing to the considerable deposit of ferric hydroxide on the original starch.

The results of the determination of phosphorus and iron contents of the amylose and the amylopectin fractions treated with ferric chloride solution followed by dialysis, suggest that the iron could combine with the ionization groups of phosphorus in the amylopectin component in addition to complex compound formation.

Since difference has been observed in the rate of staling on rheological properties during the storage-peroid between the starches prepared with redistilled water and with well water, it is supposed that metal ions in water used for preparing the starch might retard the staling of the starch granules.

Synopses of the Articles printed in NIPPON NÔGEI-KAGAKU KAISHI
(in Japanese)
Vol. 32, No. 4 (1958)

Studies on the Denaturation of Casein by Irradiation of Ultraviolet Light. Part I. The Observation of Electrophoretic Behaviors and the Destruction of the Amino Acid Residues. (p. *251*~*255*)

By Yataro OBATA and Sadao SAKAMURA
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The action of ultraviolet light on casein was studied by electrophoresis, determination of some amino acid content, measurement of UV-spectrum and degree of color development were also investigated.

A new finding was obtained, that is, in the electrophoretic pattern of whole-casein, the β-casein peak is flatened while α-casein peak remained unchanged. The same fact was also ascertained with each purified α- and β-casein.

Moreover, though α-casein was not changed electrophoreticaly, it followed the decomposition of amino acid residues (tryptophan, tyrosin, histidin) and browning was accelerated in the presence of riboflavin but retarded in the presence of ascorbic acid.

The UV-absorption curve increased in an all-over region, remarkably in the minimum region.

Thus, from the additional measurement of the amino group content and the ultracentrifuge pattern it is presumed that such a denaturation occurred without the rupture of the peptid bond of the protein.

Oxidative Metabolism of Phenylacetic Acid by *Penicillium chrysogenum* Q 176. Part XIII. Isolation and Identification of an Intermediate Metabolite of *p*-Hydroxyphenylacetic Acid. (2) (p. *256*~*259*)

By Masao ISONO
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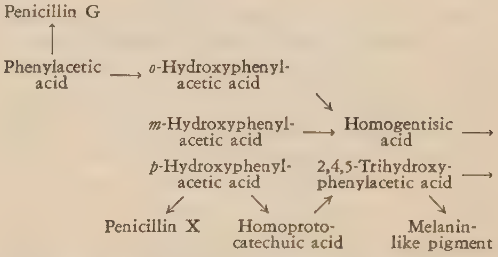
The metabolism of phenylacetic acid (PAA), *o*-, *m*- and *p*-hydroxyphenylacetic acids (*o*HPAA, *m*HPAA and *p*HPAA) was examined using a biochemical mutant "*Penicillium chrysogenum* Q 176 homogentisic acid mutant" which was induced from the normal strain by the aid of ultraviolet ray irradiation. The following results indicated the metabolic pathway of PAA and its monohydroxy derivatives to be:


Penicillin G
Phenylacetic acid
o-Hydroxyphenylacetic acid
m-Hydroxyphenylacetic acid
Homogentisic acid
p-Hydroxyphenylacetic acid
2,4,5-Trihydroxyphenylacetic acid
Penicillin X
Homoprotocatechuic acid
Melanin-like pigment

(1) PAA, *o*HPAA, *m*HPAA and *p*HPAA were all completely oxidized by the normal strain and none of their metabolic intermediates were detectable.

(2) The mutant produced and accumulated homogentisic acid (HGA) from PAA via *o*HPAA.

(3) *m*HPAA as well as *o*HPAA was converted into HGA by the mutant.

(4) *p*HPAA, added to the mutant culture, was converted into a melanin-like pigment. The formation of the melanin-like pigment from *p*HPAA was prevented by the presence of L-ascorbic acid and considerable amounts of homoprotocatechuic acid (HPCA) and 2,4,5-trihydroxyphenylacetic acid (2,4,5-THPAA) were accumulated in the mutant culture.

(5) In the presence of L-ascorbic acid, HPCA was also converted into 2,4,5-THPAA by the mutant.

Studies on *Bacillus Natto* Protease. Part VI. Crystallization of an Inhibitor of *Bacillus Natto* Protease present in Potato. (p. *260*~*262*)

By Ken WATANABE, Mitsuyoshi YOSHIKAWA, Yoshitaka NONOGUCHI and Keigo KONDO
(*Biochemical Laboratory, Hyogo University of Agriculture*)

A 1% saline extract of potato was found to have strong inhibiting effects towards both *Bacillus Natto* protease which were isolated in a crystalline state from the culture-liquid and crystalline trypsin.

The potato inhibitor of *Bacillus Natto* protease was purified and isolated as crystalline protein. As this inhibitor was ineffective to trypsin, it is not identical with the crystalline inhibitor of trypsin which K. Sohonie and K. S. Ambe isolated from potato.

This inhibitor did not show inhibiting action on pepsin and papain.

The crystalline inhibitor combined stoichiometrically with the crystalline *Bacillus Natto* protease and inactivated enzyme activity completely in a nitrogen ratio of apprx. 1:1.

Chemical Studies on Hair. Part V. Combination of Hair Protein with Acid (hydrochloric acid) and Base (sodium hydroxide). (p. *263～265*)

By Gunki FUNATSU and Masaru FUNATSU
(*Biochemical Laboratory, Faculty of Agriculture, Kyushu University*)

A study was made on the combination of human dark-hair protein with acid (HCl) and base (NaOH). In the case of natural hair protein, the maximum acid-binding capacity was 0.84 millimoles per gram and the maximum base-binding capacity was about 0.8 millimoles. It did not adsorb acid and base in the range of pH 4–8, and the isoelectric point of hair was pH 6. On the contrary, in the 30% H_2O_2 treated hair (treatment: at 30°C, 24 hours), the maximum acid-binding capacity was 0.55 milimoles, maximum base-binding capacity about 1.4 millimoles, and isoelectric point pH 5.

These facts would suggest that acidic groups (perhaps $-SO_3H$ groups) are introduced in the H_2O_2 treated hair.

Studies on the Denaturation of Proteins with Sodium-*p*-Aminosalicylate (PAS). Part VII. An Examination of the Properties of PAS Binding by Ovalbumin applying by a Surface Chemical Method. (p. *265～268*)

By Masaru FUNATSU
(*Laboratory of Biochemistry, Faculty of Agriculture, Kyushu University, Fukuoka*)

In order to ascertain PAS binding occurring inside the protein molecule, which was assumed to control the degree of denaturation[1], the properties of monolayer films formed with ovalbumin denatured by PAS were examined.

The surface pressure of monolayer film was measured by Sasaki's modification of Wilhelmi's hanging-plate method[2] at 21–24°C.

When ovalbumin was treated with different concentrations of PAS and then spread on with a 0.5 M KCl solution at pH 5.5, the limiting area of monolayer films are also increased with the increase of PAS concentration. This suggested that the binding of PAS occurs inside the protein molecule, and that protein molecules are combined more readily by hydrogen bonding with PAS than with H_2O molecules.

Moreover, since all of the FA vs F lines obtained at different concentrations of PAS joined at one point on the FA axis, the binding of PAS by protein was assumed to occur intramolecularly and not intermolecularly, i.e., PAS-denatured ovalbumin did not appear to undergo either disaggregation or aggregation.

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1) M. Funatsu, *J. Agr. Chem. Soc. Japan*, **31**, 52, 367 (1957)
2) T. Sasaki, *J. Chem. Soc. Japan*, **62**, 796 (1941).

Studies on the Cereal Starch. Part III. On the Visco-elasticity of Non-glutinous Rice Starch Paste. (p. *268～272*)

By Shinjiro CHIKUBU, Hisaya HORIUCHI and Tatsuo TANI
(*Food Research Institute, Ministry of Agriculture and Forestry*)

The authors have made studies in concern of the rheological properties of non-glutinous rice starch pastes in the past, especially in the measurement of visco-elasticity. A 8.5% water slurry of each sort of rice starch (japonica and indica) was heated by a Brabender Amylograph and the swelling of starch granule was observed under the microscope at several stages. As the result, it is supposed that the starch granule of indica rice had been rather steady in comparison with japonica rice. In regard to the visco-elasticity of a hot paste body, a remarkable difference between each starch except that of Thail rice was not recognized. Moreover, each sort of paste was cooled to 10°C and the change of visco-elasticity of cold paste body was measured time by time. As a result, it was found that the starch paste of indica rice has a greater visco-elasticity than japonica and also changes to a great extent.

Studies on Thiamine of Cereals and Beans by the Photographic Method. Part VI. The Distribution of Thiamine in Paddy Seedlings at Different Stages of Germination in the Dark. (p. *272～275*)

By Shinjiro CHIKUBU, Hisaya HORIUCHI and Shoji YANAI
(*Food Research Institute, Ministry of Agriculture and Forestry*)

Studies have been made about the distribution of thiamine in the paddy at different stages of germination (kept at 30°C, in the dark, for 14 days) by the photographic method of thiochrome fluorescence. In each stage the moisture contents, fresh-weight of one plant and the thiamine content (both total and free) were measured.

In regard to the thiamine content of each fraction in the germinated seed, a decrease was observed after 2 day's germination in the endosperm, but there was a rather rapid increase in the embryo. However, the whole thiamine content of one seed was not markedly changed through 14 day's germination.

In regard to the distribution of thiochrome fluorescence in embryo tissue, a more intensive fluorescence was observed in door tissue and scutellum than in primary leaf and root before germination, whereas, the fluorescence accumulated in primary leaf and root after 2 day's germination. As well as in the sprout, the fluorescence was also recognized heavily in the leaf-blade rather than the leaf sheath, especially strong in the buscular bundle. As for the root, there was also a high concentration of fluorescence in epidermis and central cylinder more than that in the cortex.

On the Migration Distance in Paper-Electrophoresis. Part IV. Studies on the Migration Distance and MG Value of Serum Proteins. (1)

(p. *275~279*)

By Kiyoshi SAKAMOTO and Kinuko SAITO

(*Laboratory of Nutrition, Faculty of Junior Course, Kagoshima Prefectural University*)

When the author mathematically analysed the migration distance of the normal human serum in the apparatus where evaporation from the surface of the filterpapers maintained horizontal was admitted, he found that the migration velocities of serum protein fractions showed decrease of speed approximately equal to the logarithmic decrement. Accordingly, he succeeded to calculate the net migratoin velocity of each serum protein fraction on the filterpaper by the logarithmic decrement formula availing the migration distances obtained in the above apparatus and obtained the MG value of the serum protein fraction under the following definition until it was acknowledged that these MG values became constant regardless voltage, current, time of electrophoresis, dropped points and lengths of filterpapers.

$$\mathrm{MG}=\frac{\text{migration distance of serum protein fraction from }\gamma\text{-globuline}}{\text{migration distance of serum albumine from }\gamma\text{-globuline}}$$

On the Migration Distance in Paper-Electrophoresis. Part V. Analysis of the MG Value. (1)

(p. *279~283*)

By Kiyoshi SAKAMOTO and Kinuko SAITO

(*Laboratory of Nutrition, Faculty of Junior Course, Kagoshima Prefectural University*)

As the author discovered the fact that the migration velocities of amino acids and serum proteins in the apparatus where evaporation from the surface of filterpapers kept horizontal was admitted showing decrease of speed approaching the logarithmic decrement, he attempted the mathematical analysis of the distance ratio MG values applying the fact obtained above. Thence, it could be theoretically acknowledged that these MG values, became equivalent to the ratio of differences of mobilities of the migrants, became constant regardless voltage, current, time of electrophoresis, dropped points and lengths of filterpapers.

Furthermore, it was acknowledged that MG values cannot be admitted unless parallel graphic lines are obtainable between the axis each representing the migration distance and the distance of dropped points from the center of filterpaper.

Studies on the Inhibitory Factors in Acetone-Butanol Fermentation. Part V. The Microbiological Characteristics of the Contaminants.

(p. *283~287*)

By Shigeru NEMOTO (*Gôdô-Shusei Inc.*)

The author has studied the microbiological characteristics of the four isolated contaminants. According to the ordinary classification of bacilli, these four contaminants are too closely resembled to each other so that it is impossible to classify them. When he carried out the experiments on the growth of them by the use of "pepton, aspargin, $NaNO_2$, NH_4Cl, $NaNO_3$, $(NH_4)_2SO_4$, NH_4-Lactate, and NH_4-Tartrate" as Nitrogen sources, the different characteristics of these four contaminants could be determined.

According to Bergey's manual of Determinative Bacteriology (sixth edition. 1948), the above four contaminants were classified them as a kind of variety of *Bacillus subtilis*.

Studies on the Inhibitory Factors in Acetone-Butanol Fermentation. Part VI. On the so-called "Acid-forming Fermentation" in Acetone-Butanol Fermentation. (1)

(p. *287~291*)

By Shigeru NEMOTO (*Gôdô-Shusei Inc.*)

The so-called "Acid-forming fermentation" and the other abnormal fermentation in the case of acetone-butanol fermentation were investigated. A short time after the beginning of fermentation, the movement of the mashes comes to stop and gas evolution ceases. After cessation for a while, the mashes resume fermentation, though their movement is in reverse direction. Thereafter, the titrable acidity is gradually increased and bacillus which looks like Diplococcus and bacilli rich in granule, come to appear.

In this case, the consumption of Starch is very small. After this cessation, Lactic Acid is chiefly increased and Acetic Acid is also increased in a small amount. Although Acetone and Butanol are not produced, Ethanol is increased more than twice as much as in normal fermentation.

Judging from these above facts, the fermentation after cessation can be considered to belong to participate in the scheme of Heterolactic Acid Fermentation.

Studies on the Anomalous Linkage in Starch. Part IV. Problems on Z-enzyme. (p. *291~298*)

By Akira BABA and Hidejiro KOJIMA

(*Shiga Agricultural College, Shiga Prefecture, Japan*)

A number of amylose solutions were prepared by different procedures, and the saccharifying limit of their amylose with crystalline sweet potato β-amylase were examined. On the other hand, an α-amylase action of apricot emulsin was revealed. The results of experiments are summarized as follows:

(1) Amylose, the substrate, was saccharified in a limit-value of 60–70 per cent in all cases. However, when the amylose was heated with 0.4 N sodium hydroxide at 98°C for one hour, it was saccharified completely by the action of β-amylase.

(2) The blue value of the amylose was considerably diminished by the long-term storage (3 years in a desiccator at room temperature), but its viscosity and saccharifying limit were practically invariable.

(3) It was substantiated that "Z-enzyme" action is not to be caused by β-glucosidase or β-xylosidase of apricot emulsin.

(4) "Z-enzyme" was intimated as an α-amylase.

Studies on Chitin from Standpoints of Polymer Chemistry. Part IV. Relations of the Effective Charges of Glycol Chitosan (GC) and its N-Methyl Derivative (MGC) with Interaction of the Negative Colloid. (p. *298~302*)

By Satoshi OKIMASU

(*Hiroshima Women's College*)

GC and MGC, which are water-soluble derivatives prepared from chitin by the author, have already been used as a positive colloid reagent for the method of colloid titration, which is, a new volumetric analysis of various negative colloids (pectin, alginic acid, tannin, etc.). Though colloid titration is based on a stoichiometric combination of positive and negative polymer ions, the relations of this combination with the effective charges of each polymer ion have been studied here. The conductivity measurements and the colloid titrations of both GC and MGC solutions with N/400 potassium-polyvinyl sulfonate solution (PVSK) were performed at various conditions of each solution, and mutual relations were discussed. As the results, the following two points were concluded: (1) Though the effective charges of GC and MGC ions delicately change at various conditions as in the case of ordinary polyelectrolytes, the combination reaction with PVSK is mainly controlled by the degree of ionization and is stoichiometric in a dilute solution (below ca. 0.25%) regardless of the changes of effective charges. (2) However, when the solution contains an inorganic divalent ion in the concentration of about 0.01N, this stoichiometric combination is somewhat disturbed.

Studies on Chitin from the Standpoints of Polymer Chemistry. Part V. Carboxymethylation of Chitin. (p. *303~308*)

By Satoshi OKIMASU

(*Hiroshima Women's College*)

As a method to prepare a water-soluble derivative of chitin, carboxymethylation with sodium-monochloracetate (ClAcONa) in the presence of NaOH was examined kinetically in a homogeneous dispersion of alkali chitin, including examination of the decomposition of ClAcONa with NaOH, which was a side-reaction. In this case, the degree of carboxymethylation of chitin was estimated by the method of colloid titration. As the results, the following points were concluded: (1) Activation energy of carboxymethylation is 22.4 Cal being in coincidence with that of decomposition of ClAcONa, accordingly it is impossible to inhibit the side-reaction by only controlling the temperature of reaction. (2) The concentration of NaOH in reaction medium has greater effect upon the velocity of carboxymethylation than that of the side-reaction. It is thus better for saving the reaction time as the concentration is adjusted as high as possible, as far as the reaction medium is homogeneous (usually, below ca. 20%). (3) The reaction of carboxymethylation is very fast being about twenty times of the velocity of the side-reaction. When the initial concentration of NaOH is about 15%, carboxymethylation is completed in about twelve hours at 30°. (4) Na-carboxymethyl chitosan thus obtained is easily-soluble in water, and gives a very viscous colloid solution.

Studies on Amino-carbonyl Reaction of Milk Products. Part XVI. The Mechanisms of Degradation of Lactose. (1) Isolation and Identification of Galactulose in Highly Heated Milk. (p. *309~313*)

By Susumu ADACHI and Takeo NAKANISHI
(*Laboratory of Animal Products Technology, Faculty of Agriculture, Tohoku University, Sendai*)

Two keto-sugars in addition to lactose, galactose, and glucose have been detected in highly heated milk (3 lb., 5 hrs.) by the application of paper chromatography. The spot corresponding to galactose was markedly increased, the lactose spot decreased in concentration, and the glucose spot failed to appear on the chromatogram. D-Galactose was isolated as the crystalline galactose-*o*-tolylhydrazone from the heated milk. One of the keto-sugars was purified by carbon-Celite 545 and ion exchange Amberlite IRA-410 (in $\mathrm{HSO_3}'$ form) column chromatography, and identified as lactulose from the results of phenylosazone formation, acid hydrolysis, and some physico-chemical properties of the crystals.

On Browing of Milk Caramels. (3) A Colorimetric Determination of Deterioration derived from Amino-carbonyl Reaction in Milk Caramels. (p. *313~316*)

By Susumu ADACHI
(*Laboratory of Animal Products Technology, Faculty of Agriculture, Tohoku University, Sendai*)

A modification of the Chapman and McFarlane's ferricyanide method for determining the reducing power of milk caramels is described. The increase in capacity to reduce potassium ferricyanide was accompanied with the spoilage induced from the amino-carbonyl reaction, namely, the formation of stale flavour, browning of the colour, and the shifting of pH to the acidic side in milk caramel during storage. It has been shown that the reducing substances were mainly formed from milk-reducing sugar interaction. The critical value which distinguished the fresh products from the spoilage was about 0.100~0.150 (measured as absorbance).

Studies on Amylase of Various Species of Molds. Part XIV. Hydrolysis of Taka-amylase B upon Various Substrates. (p. *316~320*)

By Hiroshi OKAZAKI
(*Takamine Research Laboratory, Sankyo Co., Ltd.*)

Hydrolysis by Taka-amylase B (a glucose-producing amylase of *Aspergillus oryzae*) was observed with various substrates.

Given a sufficient time for hydrolysis, potato starch, amylopectin, amylose, glutinous rice starch, glycogen, beta-limitdextrin, alpha-beta-limitdextrin, maltose and panose were all revealed to be hydrolysed up to 100% of the theoretical glucose. Panose hydrolytic activity of the enzyme was very faint as compared with that of gluc amylase, a glucose-producing amylase of *Rhizopus delemar*[1]. When alpha-phenylmaltoside was used as a substrate, the enzyme was revealed to split the glucosidic linkage between two glucosidic residues in the substrate and not to split the linkage between phenyl and glucosidic residues. The reverse occurs the case of Taka-amylase A[2] (alpha-amylase of *Aspergillus oryzae*).

A suggestion for a classification of glucose-producing amylases of molds into three groups was proposed. Typical examples of the three groups are, gluc amylase of *Rhizopus delemar* (easily hydrolysis starch up to 100%), Taka-amylase B (hardly hydrolyses starch up to 100%) and gamma-amylase (lacks in maltose hydrolyzing activity) respectively.

[1] Y. Tsujisaka and Fukumoto: Science and Industry (Japan), **30**, 398 (1956).

[2] T. Ikenaka, S. Matsubara, H. Toda and T. Tsugita: Presented at the Meating of the Spmposia on Enzyme Chemistry (Japan), Jan. 26, 1957.

Studies on Streptomycetes. *Streptomyces humidus* nov. sp., the source of dihydrostreptomycin. (p. *321~324*)

By Kôiti NAKAZAWA, Motoo SHIBATA, Kazuo TANABE and Hiroichi YAMAMOTO
(*Institute for Fermentation, Osaka*)

A strain of streptomycete No. 23572 has been found to produce dihydrostreptomycin. This streptomycete is characterized by its hygroscopic property and considered to be a member of the *Streptomyces hygroscopicus* group. It is therefore designated as *Streptomyces humidus* nov. sp.

Studies on Streptomycetes. On the production of Dihydrostreptomycin and Vitamin B_{12}. (p. *324~330*)

By Kôiti NAKAZAWA, Motoo SHIBATA, Hiroichi YAMAMOTO, Toshihiko KANZAKI, Eiji HIGASHIDE, Yoshio HAMADA, Akira MIYAKE and Eisuke IWASAKI
(*Institute for Fermentation, Osaka*)

For the production of dihydrostreptomycin, the combination of HCl-hydrolysate of casein and corn steep liquor was found to be the best. Galactose or fructose was the most superior carbon source but maltose, lactose and sucrose were only utilized very slightly.

When nitrogen sources such as beef extract and peptone were employed, vitamin B_{12} was scarcely produced. Soybean meal, corn steep liquor or the combination of both nutrients revealed to be most preferable. Though various vitamins and precursors had no effect Co^{++} considerably stimulated the synthesis of vitamin B_{12}.

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Vol. 22, No. 2

Page	Column	Line		
127	right	1*	*insert* Received December 24, 1957	
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Short Communications

S. SHIMIZU and N. IKEDA: The Essential Oil of *Mentha rotundifolia* of Europian Origin. (Studies on the Essential Oil of *Mentha rotundifolia*, Part 4).. 201

S. TAMURA, G. TAMURA, M. TAKAI, S. NAKAMURA and T. SHIRO: The Biological Activity of Some Homologs of Mevalonic Acid. 202

T. ISHIHARA and T. KONDO: Studies on Lignin. Part V. A Reexamination of Hydrogen Sulfide Cooking of Vanillyl Alcohol, and Conversion of Vanillyl Disulfide into Vanillyl Monosulfide. 203

Synopses of the Articles printed in NIPPON NÔGEI-KAGAKU KAISHI.. *1~12*

Errata *12*

Journal of the Agricultural Chemical Society of Japan

(Printed in Japanese)
Vol. 32, No. 3 (1958)

T. WATANABE, O. WATANABE, T. YASUNAGA, M. UEMURA and T. HORIGUCHI: The Studies on the Milling Quality of Domestic Wheat. Part II.

T. MATSUMOTO, M. TAKAHASHI, T. KOMIYAMA and M. YAMAMOTO: The Influence of Feed and Feeding upon the Ruminal Gas Formation. Part III, IV.

M. FUNATSU, G. FUNATSU, Y. IMAMURA and T. NAGAOKA: Chemical Studies on Hair. Part IV.

Y. WATANABE: Studies on the Nutritive Value of Carbohydrate of Shiitake.

K. SAKAMOTO and K. SAITO: On the Migration Distance in Paper Electrophoresis. Part II, III.

H. KOJIMA and Y. MATSUYA: Studies on the Microbiological Assay of Vitamins and Amino Acids by the Pulp Disc Method. Part V.

S. NEMOTO: Studies on the Inhibitory Factors in Acetone-Butanol Fermentation. Part III, IV.

M. ITO: Polarographic Catalytic Waves of Proteins by Tris (Ethylenediamine) Cobalt (III) Complex.

T. BEPPU, S. ABE and K. SAKAGUCHI: Accumulation of *allo-iso*-Citric Acid by Genus Penicillium. Part I.

K. MATSUSHIMA: On the Naturally Occuring Inhibitors of *Aspergillus*-protease. Part III.

K. MATSUSHIMA: Studies on the Proteolytic Enzymes of Molds. Part XIV.

M. HONGO: Butanol Fermentation. Part XVIII, XIX.

D. YONEZAWA, H. TANAKA and H. SHIMOMURA: Studies on Denatured Egg Albumin. Part I.

J. FUKUMOTO, T. YAMAMOTO and K. ICHIKAWA: Studies on Bacterial Proteinase. Part VIII, IX.

K. YAMANAKA: Studies on Pyruvate and Carbohydrate Metabolisms by Lactic Acid Bacteria. Part VII, VIII.

S. TAKAHASHI and Y. KIHARA: Rheological Studies on Potato Starch. Part II.

Journal of the Agricultural Chemical Society of Japan

(Printed in Japanese)
Vol. 32, No. 4 (1958)

Y. OBATA, and S. SAKAMURA: Studies on the Denaturation of Casein by Irradiation of Ultraviolet Light. Part I.

M. ISONO: Oxidative Metabolism of Phenylacetic Acid by *Penicillium chrysogenum* Q 176. Part XIII.

K. WATANABE, M. YOSHIKAWA, Y. NONOGUCHI and K. KONDO: Studies on *Bacillus Natto* Protease. Part VI.

G. FUNATSU and M. FUNATSU: Chemical Studies on Hair. Part V.

M. FUNATSU: Studies on the Denaturation of Proteins with Sodium-*p*-Aminosalicylate (PAS). Part VII.

S. CHIKUBU, H. HORIGUCHI and T. TANI: Studies on the Cereal Starch. Part III.

S. CHIKUBU, H. HORIUCHI and S. YANAI: Studies on Thiamine of Cereals and Beans by the Photographic Method. Part VI.

K. SAKAMOTO and K. SAITO: On the Migration Distance in Paper Electrophoresis. Part IV, V.

S. NEMOTO: Studies on the Inhibitory Factors in Acetone-Butanol Fermentation. Part V, VI.

A. BABA and H. KOJIMA: Studies on the Anomalous Linkage in Starch. Part IV.

S. OKIMASU: Studies on Chitin from the Standpoints of Polymer Chemistry. Part IV, V.

S. ADACHI and T. NAKANISHI: Studies on Aminocarbonyl Reaction of Milk Products. Part XVI.

S. ADACHI: On Browning of Milk Caramels. (3).

H. OKAZAKI: Studies on Amylases of Various Species of Molds. Part XIV.

K. NAKAZAWA, M. SHIBATA, K. TANABE, H. YAMAMOTO, T. KANZAKI, E. HIGASHIDE, Y. HAMADA, A. MIYAKE and E. IWASAKI: Studies on Streptomycetes.

Review

L. A. UNDERKOFLER: Some Modern Developments in the Fermentation Industries in the United States.

Technical Article

T. NISHIKAWA: Recent Movement on Formula Feed.

PRINTED BY
KASAI PUBLISHING & PRINTING CO.
(Pan-Pacific Press)
MINATO-KU, TOKYO